Europäisches Patentamt	Eur pean Patent Office	Office eur péen des brevets

CA00/642

4

Bescheinigung | Certificate | Attestation

Die angehefteten Unterlagen stimmen mit der ursprünglich eingereichten Fassung der auf dem nächsten Blatt bezeichneten europäischen Patentanmeldung überein.

The attached documents are exact copies of the European patent application described on the following page, as originally filed.

Les documents fixés à cette attestation sont conformes à la version initialement déposée de la demande de brevet européen spécifiée à la page suivante.

Patentanmeldung Nr. | Patent application No. | Demande de brevet n°

99201745.9

PRIORITY DOCUMENT
SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

Der Präsident des Europäischen Patentamts:
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
p.o.

I.L.C. HATTEN-HECKMAN

DEN HAAG, DEN
THE HAGUE, 20/06/00
LA HAYE, LE

EPA/EPO/OEB Form 1014 - 02.91

Europäisches Patentamt
European Patent Office
Office eur péen des brevets

Blatt 2 der Bescheinigung
Sheet 2 of the certificate
Page 2 de l'attestation

Anmeldung Nr.:
Application no.:
Demande n°:
99201745.9

Anmeldetag:
Date of filing:
Date de dépôt:
02/06/99

Anmelder:
Applicant(s):
Demandeur(s):
CENTRUM VOOR PLANTENVEREDELINGS- EN REPRODUKTIEONDERZOEK
6708 PB Wageningen
NETHERLANDS
Her Majesty in Right of Canada, represented by The Minister of Agriculture and Agri-Food Canada
Ottawa, Ontario K1A 0C6
CANADA

Bezeichnung der Erfindung:
Title of the invention:
Titre de l'invention:
Use of the BNM3 transcriptional activator to control plant embryogenesis and regeneration processes

In Anspruch genommene Priorität(en) / Priority(ies) claimed / Priorité(s) revendiquée(s)

Staat: State: Pays:	Tag: Date: Date:	Aktenzeichen: File no. Numéro de dépôt:

Internationale Patentklassifikation:
International Patent classification:
Classification internationale des brevets:
C12N15/29, C12N15/82, C12N5/10, C07K14/415, A01H5/00, A01H5/10

Am Anmeldetag benannte Vertragstaaten:
Contracting states designated at date of filing: AT/BE/CH/CY/DE/DK/ES/FI/FR/GB/GR/IE/IT/LI/LU/MC/NL/PT/SE
Etats contractants désignés lors du depôt:

Bemerkungen:
Remarks:
Remarques:

USE OF THE *BNM3* TRANSCRIPTIONAL ACTIVATOR TO CONTROL PLANT EMBRYOGENESIS AND REGENERATION PROCESSES

The present invention relates to asexual embryo formation and regeneration in plants. More specifically, it relates to processes for producing asexually-derived embryos, and for enhancing regeneration capacity in plants. The present invention also relates to heterologous protein production systems in plants, and the uses thereof.

BACKGROUND OF THE INVENTION

A typical angiosperm seed consists of three major components, the embryo, the endosperm and the maternal seed coat. Seed development begins with a double fertilization event, in which one sperm cell nucleus fuses with the egg cell nucleus to form the embryo, and a second sperm cell nucleus fuses with two central cell nuclei to form the endosperm. Embryo development itself can be separated into three developmental phases. The first phase of embryo development is one of cell division and morphogenesis, which serves to establish the major tissue types and organ systems of the mature plant. The second phase encompasses a period of rapid cell expansion and is characterized by the synthesis of storage reserves that sustain the embryo during germination and early seedling development. In the final phase of embryo development, the embryo becomes desiccated and enters into a period of developmental arrest or dormancy. All of the above events normally take place while the seed remains attached to the maternal plant.

Many plant species are capable of producing embryos in the absence of fertilization. This process of asexual embryo development may occur naturally, for example on the leaf margins of *Bryophyllum* (Yarborough, 1923) and *Malaxis* (Taylor, 1967), or within the ovule of apomictic plants (Koltunow, 1995). Apomixis refers to the production of a seed from the maternal ovule tissues in the absence of egg cell fertilization. Asexual embryo development may also be induced *in vitro* from gametophytic or somatic tissue (Mordhorst *et al.*, 1997) or, as shown recently, may be

induced by genetic modification of gene expression (Ogas *et al.*, 1997; Lotan *et al.*, 1998).

Three major mechanisms of apomixis, diplospory, apospory and adventitious embryony, have been observed. Each mechanism differs with respect to the source of the cell that gives rise to the embryo and with respect to the time during ovule development at which the apomictic process is initiated. Diplospory and apospory are considered gametophytic forms of apomixis as they involve the formation of diploid embryo sacs. Adventitious embryony does not involve the production of a mitotically-derived embryo sac.

In diplospory, the megaspore mother cell does not undergo normal meiosis, but rather divides mitotically to produce a diploid embryo sac instead of the normal haploid embryo sac. One of the cells of the embryo sac functions as the egg cell and divides parthenogenetically (without fertilization) to form an embryo. In some species the unreduced polar nuclei of the embryo sac may fuse to form the endosperm (autonomous endosperm production), the nutritive tissue of the seed, while in other species pollination is necessary for endosperm production (pseudogamy).

In aposporous apomicts, parthenogenic embryos are produced from additional cells, the aposporous initials, that differentiate from the nucellus. As with the megagametophyte of diplosporous species, the aposporous initial undergoes mitotic divisions to produce a diploid embryo sac. Aposporous embryos are not derived from the megagametophyte and can therefore co-exist within a single ovule with sexually-derived embryos. Autonomous production of endosperm is rare in aposporous species. Aposporous apomicts therefore depend on fertilization of the polar nuclei of a meiotically-derived embryo sac for the production of endosperm.

With adventitious embryony, embryos are formed directly from sporophytic ovule tissue, such as the integuments or nucellus, via parthenogenesis. Seeds derived from species exhibiting adventitious embryony generally contain multiple asexually-derived

embryos and may also contain a single sexually-derived embryo. Plants exhibiting adventitious embryo also rely on the presence of a meiotically-derived embryo sac within the same ovule for endosperm formation.

In most plant species, the apomictic trait appears to be under the control of a single dominant locus. This locus may encode one or more developmental regulators, such as transcription factors, that in sexually reproducing plants function to initiate gene expression cascades leading to embryo sac and/or embryogenesis, but which are heterochronically or ectopically expressed in apomictic plants (Peacock, 1992; Koltunow, 1993; Koltunow et al, 1995).

Apomixis is a valuable trait for crop improvement since apomictic seeds give rise to clonal offspring and can therefore be used to genetically fix hybrid lines. The production of hybrid seed is a labour intensive and costly procedure as it involves maintaining populations of genetically pure parental lines, the use of separate pollen donor and male-sterile lines, and line isolation. Production of seed through apomixis avoids these problems in that once a hybrid has been produced, it can be maintained clonally, thereby eliminating the need to maintain and cross separate parental lines. The use of apomictic seed also provides a more cost effective method of multiplying vegetatively-propagated crops, as it eliminates the use of cuttings or tissue culture techniques to propagate lines, reduces the spread of diseases which are easily transmitted through vegetatively-propagated tissues, and in many species reduces the size of the propagule leading to lower shipping and planting costs.

Although apomixis occurs in a wide range of plant species, few crop species are apomictic. Attempts to introduce apomictic traits into crop species by introgression from wild relatives (Ozias-Akins, *et al.*, 1993; WO 97/10704; WO 97/11167) or through crosses between related, but developmentally divergent sexual species (WO 98/33374), have not yielded marketable products. Other approaches have focused on the identification of gene sequences that may be used to identify or manipulate apomictic

processes (WO 97/43427; WO 98/36090), however these approaches have not led to methods for the routine production of apomictic plants.

Mutagenesis approaches have also been attempted to convert sexually reproducing plants such as *Arabidopsis thaliana* (arabidopsis) into apomictic plants (Peacock *et al.*, 1995). For example, a number of recessive "fertilization-independent seed" (*fis*) mutants have been identified that initiate partial embryo and/or endosperm at a low frequency in the absence of fertilization (Chaudhury *et al.*, 1997). However, a number of additional parameters need to be modified in order to obtain true diploid apomictic seed using *fis* mutants.

Asexually-derived embryos can be induced to form in culture from many gametophytic and somatic plant tissues (Yeung, 1995). Somatic embryos can be obtained from culture of somatic tissues by treating them with plant growth regulators, such as auxins, or auxins in combination with cytokinins. Embryos can also be induced to form in culture from the gametophytic tissues of the ovule (gynogenesis) and the anther (androgenesis, pollen or microspore embryogenesis), either by the addition of plant growth regulators or by a simple stress treatment.

Several mutants have been identified that may be used to induce efficient production of embryos *in vitro*. These include recessive arabidopsis mutants with altered shoot meristems, for example *primordia timing (pt)*, *clavata (clv)1* and *clv3*, which were shown to enhance embryogenic callus formation when seedlings were germinated in the presence of auxin (Mordhorst *et al.*, 1998). The altered expression of two arabidopsis genes, *LEAFY COTYLEDON* (*LEC1*; WO 98/37184, Lotan *et al.*, 1998) and *pickle*, have been shown to promote the production of somatic embryos in the absence of added growth regulators. The *LEC1* gene encodes a homologue of the HAP3 subunit of a CCAAT box-binding transcription factor (CBF). The *LEC1* gene controls many aspects of zygotic embryo development including desiccation tolerance and cotyledon identity. Ectopic over-expression of the *LEC1* gene in a *lec1* mutant background results in the production of 2 transgenic lines that occasionally form embryo-like structures on leaves.

These embryo-like structures express genes, such as those encoding seed storage proteins and oil body proteins, which are normally preferentially expressed in developing embryos. Plants containing a recessive mutant *PICKLE* gene produce a thickened, primary root meristem. Mutant *pickle* roots produce embryo-forming callus when the root tissue is separated from the rest of the plant and placed on minimal medium without growth regulators (Ogas *et al.*, 1997). Mutant *pickle* roots show morphological characteristics of developing seeds, such as oil bodies and, as with *LEC1* over-expressers, accumulate genes preferentially expressed in developing seeds.

Efficient production of apomictic seed is only likely to be realised through the identification and subsequent modification of developmental regulators, such as transcription factors, that are known to activate gene expression cascades leading to embryogenesis in both sexually-reproducing and apomictic plants. The present invention addresses this need by providing methods for the production of apomictic seeds comprising ectopic over-expression of an embryo-expressed AP2 domain containing transcription factor, BNM3.

SUMMARY OF THE INVENTION

The present invention relates to asexual embryo formation and regeneration in plants. More specifically, it relates to processes for producing asexually-derived embryos, and for enhancing regeneration capacity in plants.

According to the present invention there is provided an isolated DNA molecule comprising a nucleotide sequence that hybridizes to SEQ ID NO:5 under stringent conditions, that comprises at least 23 contiguous nucleotides of SEQ ID NO:5, or that is at least 70% homologous with the nucleotide sequence defined by SEQ ID NO:5.

This invention further relates to an isolated DNA molecule that hybridizes to SEQ ID NO:5 under stringent conditions, comprising a nucleic acid sequence encoding a protein, wherein the protein when present at a sufficient level within a plant cell renders the cell embryogenic, increases the regenerative capacity of the plant cell, or both renders the cell embryogenic and increases the regenerative capacity of the plant cell. Included within the present invention is the above isolated DNA molecule comprising a nucleotide sequence that hybridizes to nucleotides 1620-4873 of SEQ ID NO:5 under stringent conditions, or to the nucleotide sequence as defined within SEQ ID NO:1 or 3 under stringent conditions. Also included within the present invention is a vector comprising the isolated DNA molecule as defined above, wherein the isolated DNA molecule is under control of a regulatory element that directs expression of said DNA in a plant cell. The regulatory element may be a constitutive, inducible, tissue specific or a developmental active, regulatory element.

This invention also embraces a transformed plant cell, a transformed plant, or seed obtained from a transformed plant, each comprising the vector as defined above

This invention relates to an isolated protein encoded by an isolated DNA molecule that hybridizes to the nucleotide sequence defined by SEQ ID NO:5 under stringent conditions, wherein the protein, when present at a sufficient level within a plant

cell renders the cell embryogenic, or increases the regenerative capacity of the plant cell. Also included is a protein encoded by an isolated DNA molecule that hybridizes to nucleotides 1620-4873 of SEQ ID NO:5 under stringent conditions, or to the nucleotide sequence as defined within SEQ ID NO:1 or 3 under stringent conditions. This invention also embraces an isolated DNA molecule that encodes a protein as defined by SEQ ID NO:2 or SEQ ID NO:4. The invention also pertains to a protein comprising at least 70% homology with the amino acid sequence of SEQ ID NO:2 or SEQ ID NO:4, or comprises from about 30 to about 541 amino acids of the sequence disclosed in SEQ ID NO:2, or comprises from about 30 to about 561 amino acids of the sequence disclosed in SEQ ID NO:4.

The present invention is also directed to a method of producing asexually derived embryos comprising:

i) transforming a plant cell with a vector comprising an isolated DNA molecule that hybridizes to SEQ ID NO:5 under stringent conditions and which encodes a protein that when present at a sufficient level within the plant cell renders the plant cell embryogenic, or increases the regenerative capacity of the plant cell, or that comprises a nucleotide sequence that hybridizes to nucleotides 1620-4873 of SEQ ID NO:5 under stringent conditions, or to the nucleotide sequence as defined within SEQ ID NO:1 or 3 under stringent conditions;

ii) growing the plant cell to produce transformed tissue;

iii) selecting the transformed tissue for occurrence of the isolated DNA molecule; and

iv) assaying the transformed tissue for asexual embryo formation.

This invention also relates to the above method where the step of assaying (step iv)) involves assaying for somatic embryos, gametophytically-derived embryos, adventitious embryony, diplospory, or for haploid parthenogenesis of the embryo sac.

The present invention also embraces a method of producing an apomictic plant comprising:

i) transforming a plant cell with a vector comprising an isolated DNA molecule that hybridizes to SEQ ID NO:5 under stringent conditions and which encodes a protein that when present at a sufficient level within said plant cell renders the plant cell embryogenic, or increases the regenerative capacity of the plant cell, or that comprises a nucleotide sequence that hybridizes to nucleotides 1620-4873 of SEQ ID NO:5 under stringent conditions, or to the nucleotide sequence as defined within SEQ ID NO:1 or 3 under stringent conditions;

ii) selecting the transformed plant for occurrence of the isolated DNA molecule; and

iii) assaying the transformed plant for asexual embryo formation.

This invention also relates to the above method where the step of assaying (step iii)) involves assaying for asexually-derived embryos, somatic embryos, gametophytically-derived embryos, adventitious embryony, diplospory, or for haploid parthenogenesis of the embryo sac.

The present invention is also directed to a method of producing asexually derived embryos comprising:

i) transiently transforming a plant cell with a vector comprising an isolated DNA molecule that hybridizes to SEQ ID NO:5 under stringent conditions and which encodes a protein that when present at a sufficient level within the plant cell renders the plant cell embryogenic, or increases the regenerative capacity of the plant cell, or that comprises a nucleotide sequence that hybridizes to nucleotides 1620-4873 of SEQ ID NO:5 under stringent conditions, or to the nucleotide sequence as defined within SEQ ID NO:1 or 3 under stringent conditions;

ii) growing the transiently transformed plant cell to produce transiently transformed tissue;

iii) assaying the transiently transformed tissue for asexual embryo formation.

This invention is directed to the above method where the step of assaying (step iii)) involves assaying for asexually-derived embryos, somatic embryos, gametophytically-

derived embryos, adventitius embryony, diplospory, or for haploid parthenogenesis of the embryo sac.

The present invention also presents a method of modifying the regenerative capacity of a plant comprising

i) transforming a plant cell with a vector comprising an isolated DNA molecule that hybridizes to SEQ ID NO:5 under stringent conditions and which encodes a protein that when present at a sufficient level within the plant cell renders the plant cell embryogenic, or increases the regenerative capacity of said plant cell, or that comprises a nucleotide sequence that hybridizes to nucleotides 1620-4873 of SEQ ID NO:5 under stringent conditions, or to the nucleotide sequence as defined within SEQ ID NO:1 or 3 under stringent conditions;

ii) growing the transformed plant cell to produce transformed tissue; and

iii) assaying the transformed tissue for enhanced regeneration as compared to wild type tissue.

This invention also embraces the above method wherein step iii) includes assaying in the absence of a growth regulator.

The present invention also relates to a method of modifying the regenerative capacity of a plant comprising;

i) transiently transforming a plant cell with a vector comprising an isolated DNA molecule that hybridizes to SEQ ID NO:5 under stringent conditions and which encodes a protein that when present at a sufficient level within the plant cell renders the plant cell embryogenic, or increases the regenerative capacity of the plant cell, or that comprises a nucleotide sequence that hybridizes to nucleotides 1620-4873 of SEQ ID NO:5 under stringent conditions, or to the nucleotide sequence as defined within SEQ ID NO:1 or 3 under stringent conditions;

ii) growing the transiently transformed plant cell to produce transiently transformed tissue;

iii) assaying the transformed tissue for enhanced regeneration as compared to wild type tissue.

This invention also embraces the above method wherein step iii) includes assaying in the absence of a growth regulator.

The present invention also relates to a method of selecting a transformed plant comprising;

i) transforming a normally non-regenerative plant with a vector comprising an isolated DNA molecule that hybridizes to SEQ ID NO:5 under stringent conditions and which encodes a protein that when present at a sufficient level within the plant cell renders the plant cell embryogenic, or increases the regenerative capacity of said plant cell, or that comprises a nucleotide sequence that hybridizes to nucleotides 1620- 4873 of SEQ ID NO:5 under stringent conditions, or to the nucleotide sequence as defined within SEQ ID NO:1 or 3 under stringent conditions; and

ii) determining whether the transformed plant is able to regenerate under conditions in which the normally non-regenerative plant does not regenerate.

The present invention is also directed to an isolated DNA molecule comprising a DNA sequence that hybridizes to nucleotides 1-1619 of SEQ ID NO:5 under stringent conditions, or that comprises at least 22 contiguous nucleotides within nucleotides 1-1619 of SEQ ID NO:5. Also included within the scope of the present invention is a vector comprising the isolated DNA molecule as just defined, operably associated with a gene of interest, wherein the isolated DNA molecule directs the expression of the gene of interest within a plant cell. The gene of interest may be heterologous with respect to the isolated DNA molecule. The gene of interest may be selected from the group consisting of a pharmaceutically active protein, antibody, industrial enzyme, protein supplement, nutraceutical, storage protein, animal feed and animal feed supplement. This invention

also includes a transformed plant cell, a transformed plant, or seed obtained from the transformed plant, comprising the vector as just defined.

Furthermore, the present invention includes a method for directing the expression of a gene of interest within a developing embryo of a plant comprising transforming said plant with a vector containing an isolated DNA molecule that hybridizes to nucleotides 1-1619 of SEQ ID NO:5 under stringent conditions, or that comprises at least 22 contiguous nucleotides within nucleotides 1-1619 of SEQ ID NO:5.

This invention also pertains to a method of producing a protein of interest comprising

i) transforming a plant with at least one vector, comprising an isolated DNA molecule that hybridizes to SEQ ID NO:5 under stringent conditions and which encodes a protein that when present at a sufficient level within the plant cell renders the plant cell embryogenic, or increases the regenerative capacity of said plant cell, or that comprises a nucleotide sequence that hybridizes to nucleotides 1620-4873 of SEQ ID NO:5 under stringent conditions, or to the nucleotide sequence as defined within SEQ ID NO:1 or 3 under stringent conditions to produce a transformed plant;

ii) selecting the transformed plant for occurrence of the isolated DNA molecule; and

~~iv) growing the transformed plant in order to produce the protein of interest,~~

wherein expression of the protein of interest is induced by the expression product of said isolated DNA.

This method may also comprise transforming the plant with a second vector comprising a nucleotide sequence encoding the protein of interest under the control of a regulatory element, wherein the regulatory element induced by the expression product of the isolated DNA. Furthermore, this method may also be used to produce a protein of interest wherein the protein of interest is a native protein.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other features of the invention will become more apparent from the following description in which reference is made to the appended drawings wherein:

Figure 1 shows a schematic representation of the effect of culture temperature on the developmental fate of isolated microspores and pollen of *Brassica napus*. Late uninucleate microspores and early binucleate pollen cultured at 25 °C or lower continue to divide and form functional pollen grains (gametophytic), while the same microspores and pollen cultured at 32 °C undergo numerous sporophytic divisions, leading to the formation of haploid embryos (embryogenic). Late uninucleate microspores and early binucleate pollen cultured for one day at 25 °C, followed by culture at 32 °C may undergo gametophytic divisions, but form neither embryos nor mature pollen grains (non-embryogenic).

Figure 2 shows the alignment of the DNA sequences depicted in SEQ ID NO:1 and SEQ ID NO:3. The ATG and TAG translation initiation and translation termination codons are shown in bold. Identical nucleotides are indicated by (*) and gaps are indicated by (-).

Figure 3 shows the alignment of the predicted protein sequences encoded by the DNA of SEQ ID NO:1 and SEQ ID NO:3. The amino acid sequence of the first AP2 domain repeat (repeat 1) and the second AP2 domain repeat (repeat 2), are shown in bold. Identical amino acids are indicated by an asterisk (*) and mismatches by a dot (.) below the sequence alignment.

Figure 4 shows the presence of two *BNM3* genes in the *Brassica napus* genome. A DNA gel blot containing restriction digests of *B. napus* c.v. Topas genomic DNA was hybridized to a *BNM3A* cDNA fragment under high stringency conditions. The *BNM3*A cDNA hybridizes to two DNA fragments under these conditions. These

fragments correspond to the *BNM3A* and *BNM3B* genes. The position of the molecular size markers (Lambda DNA *Hind* III restriction fragments) is indicated to the left the figure. The restriction enzymes used to digest the DNA are indicated above the blot.

Figure 5 shows the alignment of the predicted protein sequence encoded by the DNA of SEQ ID NO.1 (BNM3A) with the predicted protein sequences of other AP2 domain proteins. The amino acid sequence of BNM3A, beginning at position 208, and spanning the first AP2 domain repeat (AP2 domain repeat 1), the second AP2 domain repeat (AP2 domain repeat 2), and the linker region lying between the two repeats (linker), was aligned with the amino acid sequence of other proteins containing two AP2 domains. The amino acid similarity in this region ranges from 53% for APETALA2 to 80% for ZMMHCF1. Identical amino acids are indicated by (*) and gaps are indicated by (-). Protein names are indicated on the left and are abbreviated as follows: ANT, AINTEGUMENTA (accession number U41339); ZM, ZMMHCF1 (accession number Z47554); GL15, GLOSSY15 (accession number U41466); AP2, APETALA2 (accession number U12546).

Figure 6 shows the results of gel blot analysis with a *BNM3A* cDNA fragment performed on RNA extracted from the indicated tissues. RNA gel blots contain either 5 µg (a) or 20 µg (b, c) of total RNA. Figure 6A shows the pattern of *BNM3* expression in microspore-embryo cultures. RNA was isolated from late uninucleate microspores and early binucleate pollen at the time of collection (pollen 0d), after four days in culture at 32 ° C (+ embryo), after four days in culture at 25 ° C (pollen 4d), after one day of culture at 25 ° C, followed by three days of culture at 32 ° C (- embryo) and microspore-derived embryos at the globular, heart, torpedo, 21 day old cotyledon (21 d cot), 28 day old cotyledon (28 d cot) and 42 day old cotyledon (42 d cot) stage of development. *BNM3* expression is detected in embryogenic microspores and developing microspore-derived embryos, but is absent from developing microspores and pollen collected prior to tissue culture and in non-embryogenic samples. The exposure time was seven days. Figure 6B

shows that *BNM3* gene expression is detected in developing seeds. Seeds were collected at various days after pollination (DAP). These points in development correspond approximately to the globular (7 d), heart (14 d), torpedo (18 d), early cotyledon (21 d), mid cotyledon (28 d, 35 d) and late cotyledon (42 d) stages of development. The exposure time was 14 days. Figure 6C shows that *BNM3* gene expression is not detected in non-seed tissues. Roots and leaves were collected from 14 day old greenhouse grown plants. Entire flowers as well as excised anthers and pistils were collected from opened flower buds just prior to anthesis. Small and large buds refer to closed flower buds of less than 5 mm or greater than 5 mm in length, respectively. Siliques were collected 16 days after pollination. The exposure time was 14 days.

Figure 7 shows the phenotype of *Brassica napus* and arabidopsis plants transformed with constructs containing the *BNM3* gene under control of a modified *POLYUBIQUITIN* promoter (B) and double enhanced *35S* promoter containing an AMV translational enhancer (A, C-E). Figure 7A shows embryo structures on the leaf margin of a *Brassica* T1 seedling. Figure 7B shows embryo structures on the petiole of an arabidopsis T2 seedling. Figure 7C shows embryo structures on the cotyledon of an arabidopsis T1 seedling. Figure 7D shows a scanning electron micrograph of the abaxial side of an arabidopsis T1 cotyledon. Note the bipolar nature of the embryos, as well as the emergence of a secondary embryo from the surface of a primary embryo (asterisk). Figure 7E shows a semi-thin section through one of the cotyledons of the T1 seedling shown in (Figure 7C). Note the presence of all the major organs and tissue elements of embryo, as well as the development of new embryos on the flanks of the shoot apical meristems and the cotyledons.

Figure 8 shows the increased regenerative capacity of arabidopsis plants transformed with a construct containing the *BNM3B* gene under control of a modified *POLYUBIQUITIN* promoter. Figure 8A shows wild-type and transgenic leaf and hypocotyl explants on medium containing growth regulators. Figure 8B shows

wild-type and transgenic roots on medium containing growth regulators. Figure 8C shows wild-type and transgenic leaf and hypocotyl explants on medium without growth regulators. Figure 8D shows wild-type and transgenic root explants on medium without growth regulators

DESCRIPTION OF PREFERRED EMBODIMENT

The present invention relates to asexual embryo formation and regeneration in plants. More specifically, it relates to processes for producing asexually-derived embryos, and for enhancing regeneration capacity in plants. The present invention also relates to heterologous protein production systems in plants, and the uses thereof.

Genes preferentially expressed during the induction of *Brassica napus* c.v. Topas microspore embryogenesis were isolated via subtractive screening. Seven independent cDNA clones, comprising six unique DNA sequences were found to be differentially expressed between cDNA libraries prepared from embryogenic and non-embryogenic microspore cultures. Several of these *BNM* (for *Brassica napus* microspore embryo) clones, *BNM3A* (SEQ ID NO:1) and *BNM3B* (SEQ ID NO:3), were characterized as described herein. *BNM3A* and *BNM3B* encode the amino acid sequences disclosed in SEQ ID NO:2, and SEQ ID NO:4, respectively. The genomic sequence of *BNM3A* (SEQ ID NO:5), including the regulatory region (nucleotides 1-1619 of SEQ ID NO:5), was also obtained.

"Regeneration", as used herein, refers to a morphogenetic response that results in the production of new tissues, organs, embryos, whole plants or fragments of whole plants that are derived from a single cell, or a group of cells. Regeneration may proceed indirectly via a callus phase or directly, without an intervening callus phase. "Regenerative capacity" refers to the ability of a plant cell to undergo regeneration.

By "embryogenic cell", it is meant a cell that has completed the transition from either a somatic or a gametophytic cell to a state where no further applied stimuli are necessary to produce a somatic or gametophytic embryo, respectively.

By "regulatory element" it is meant those that include developmentally regulated, tissue specific, inducible and constitutive regulatory elements. A regulatory element that is developmentally regulated, or controls the differential expression of a gene under its

control, is activated within certain organs or tissues of an organ at specific times during the development of that organ or tissue. However, some regulatory elements that are developmentally regulated may preferentially be active within certain organs or tissues at specific developmental stages, they may also be active in a developmentally regulated manner, or at a basal level in other organs or tissues within the plant as well, such regulatory elements are considered "tissue specific". Regulatory elements may be found either upstream, within, downstream, or a combination thereof, of the coding region of a gene.

An inducible regulatory element is one that is capable of directly or indirectly activating transcription of one or more DNA sequences or genes in response to an inducer. In the absence of an inducer the DNA sequences or genes will not be transcribed. Typically the protein factor, that binds specifically to an inducible regulatory element to activate transcription, is present in an inactive form which is then directly or indirectly converted to the active form by the inducer. The inducer can be a chemical agent such as a protein, metabolite, growth regulator, herbicide or phenolic compound or a physiological stress imposed directly by heat, cold, salt, or toxic elements or indirectly through the action of a pathogen or disease agent such as a virus. A plant cell containing an inducible regulatory element may be exposed to an inducer by externally applying the inducer to the cell or plant such as by spraying, watering, heating or similar methods.

A constitutive regulatory element directs the expression of a gene throughout the various parts of a plant and continuously throughout plant development. Examples of known constitutive regulatory elements include promoters associated with the CaMV 35S transcript. (Odell et al., 1985, *Nature*, **313**: 810-812), the rice actin 1 (Zhang et al, 1991, *Plant Cell*, **3**: 1155-1165) and triosephosphate isomerase 1 (Xu et al, 1994, *Plant Physiol.* **106**: 459-467) genes, the maize ubiquitin 1 gene (Cornejo et al, 1993, *Plant Mol. Biol.* **29**: 637-646), the *Arabidopsis* ubiquitin 1 and 6 genes (Holtorf et al, 1995, *Plant Mol. Biol.* **29**: 637-646), and the tobacco translational initiation factor 4A gene (Mandel et al, 1995 *Plant Mol. Biol.* **29**: 995-1004).

By "gene of interest" it is meant any gene that is to be expressed in a transformed plant. Such a gene of interest may include, but is not limited to, a gene that encodes a pharmaceutically active protein, for example growth factors, growth regulators, antibodies, antigens, their derivatives useful for immunization or vaccination and the like. Such proteins include, but are not limited to, interleukins, insulin, G-CSF, GM-CSF, hPG-CSF, M-CSF or combinations thereof, interferons, for example, interferon-α, interferon-ß, interferon-τ, blood clotting factors, for example, Factor VIII, Factor IX, or tPA or combinations thereof. A gene of interest may also encode an industrial enzyme, protein supplement, nutraceutical, or a value-added product for feed, food, or both feed and food use. Examples of such proteins include, but are not limited to proteases, oxidases, phytases chitinases, invertases, lipases, cellulases, xylanases, enzymes involved in oil biosynthesis etc. Other protein supplements, nutraceuticals, or a value-added products include native or modified seed storage proteins and the like.

The present invention is further directed to a chimeric gene construct containing a DNA of interest operatively linked to a regulatory element of the present invention. Any exogenous gene, or gene of interest, can be used and manipulated according to the present invention to result in the expression of the exogenous gene.

The activation of the expression of a gene of interest may also be under the control of a regulatory element that itself is activated by a BNM3 protein. For example, which is not to be considered limiting, a gene of interest may be fused to the napin promoter, and the napin promoter may be induced by BNM3. Furthermore, a gene of interest may be expressed within somatic tissues under the control of one or more regulatory elements induced by BNM3, so that, as will be described in more detail below, the somatic tissue develops into a seed-like structure comprising embryogenic cells, and these seed-like structures produce the products of the gene of interest.

The chimeric gene construct of the present invention can further comprise a 3' untranslated region. A 3' untranslated region refers to that portion of a gene comprising a DNA segment that contains a polyadenylation signal and any other regulatory signals

capable of effecting mRNA processing or gene expression. The polyadenylation signal is usually characterized by effecting the addition of polyadenylic acid tracks to the 3' end of the mRNA precursor. Polyadenylation signals are commonly recognized by the presence of homology to the canonical form 5' AATAAA-3' although variations are not uncommon. Examples of suitable 3' regions are the 3' transcribed non-translated regions containing a polyadenylation signal of *Agrobacterium* tumor inducing (Ti) plasmid genes, such as the nopaline synthase (*Nos* gene) and plant genes such as the soybean storage protein genes and the small subunit of the ribulose-1, 5-bisphosphate carboxylase (ssRUBISCO) gene. The 3' untranslated region from the structural gene of the present construct can therefore be used to construct chimeric genes for expression in plants.

The chimeric gene construct of the present invention can also include further enhancers, either translation or transcription enhancers, as may be required. These enhancer regions are well known to persons skilled in the art, and can include the ATG initiation codon and adjacent sequences. The initiation codon must be in phase with the reading frame of the coding sequence to ensure translation of the entire sequence. The translation control signals and initiation codons can be from a variety of origins, both natural and synthetic. Translational initiation regions may be provided from the source of the transcriptional initiation region, or from the structural gene. The sequence can also be derived from the regulatory element selected to express the gene, and can be specifically modified so as to increase translation of the mRNA.

To aid in identification of transformed plant cells, the constructs of this invention may be further manipulated to include plant selectable markers. Useful selectable markers include enzymes which provide for resistance to an antibiotic such as gentamycin, hygromycin, kanamycin, and the like. Similarly, enzymes providing for production of a compound identifiable by colour change such as *GUS* (β-glucuronidase), fluorescence, or luminescence, such as luciferase are useful.

Also considered part of this invention are transgenic plants containing a gene or chimeric gene construct of the present invention comprising a *BNM3* gene, a regulatory

element obtained from *BNM3*, or the coding region from *BNM3* in operative association with a constitutive, developmental or inducible regulatory element, or a combination thereof. Methods of regenerating whole plants from plant cells are known in the art. In general, transformed plant cells are cultured in an appropriate medium, which may contain selective agents such as antibiotics, where selectable markers are used to facilitate identification of transformed plant cells. Once callus forms, shoot formation can be encouraged by employing the appropriate plant hormones in accordance with known methods and the shoots transferred to rooting medium for regeneration of plants. The plants may then be used to establish repetitive generations, either from seeds or using vegetative propagation techniques. The constructs of the present invention can be introduced into plant cells using Ti plasmids, Ri plasmids, plant virus vectors, direct DNA transformation, micro-injection, electroporation, biolistics etc. For reviews of such techniques see for example Weissbach and Weissbach, *Methods for Plant Molecular Biology*, Academy Press, New York VIII, pp. 421-463 (1988); Geierson and Corey, *Plant Molecular Biology*, 2d Ed. (1988); and Miki and Iyer, Fundamentals of Gene Transfer in Plants. In *Plant Metabolism*, 2d Ed. DT. Dennis, DH Turpin, DD Lefebrve, DB Layzell (eds), Addison Wesly, Langmans Ltd. London, pp. 561-579 (1997). The present invention further includes a suitable vector comprising the gene or the chimeric gene construct.

A class of genes have been isolated from *Brassica napus* microspore embryo cultures. These genes have been found to be important regulators of embryogenesis by their ability to induce the formation of asexually derived embryos when ectopically expressed in the vegetative tissues of plants. These genes are hereinafter indicated as *BNM3* genes (*Brassica napus* microspore embryo). SEQ ID NO. 1 depicts the cDNA of *BNM3A* , SEQ ID NO. 3 depicts the cDNA of *BNM3B*, and the genomic sequence for *BNM3A* is given in SEQ ID NO:5. The promoter of BNM3A lies within nucleotides 1-1619 of SEQ ID NO:5. The predicted protein sequences encoded by the DNAs of SEQ ID NO. 1 and 3 are outlined in SEQ ID NOs. 2 and 4, respectively.

By *"BNM3"* or *"BNM3* gene", it is meant the sequence of oligonucleotides as disclosed in SEQ ID NOs:1, 3 or 5, or fragments, derivatives, or mutations thereof, or oligonucleotide sequences that exhibit at least 70% homology or similarity, with a

fragment or derivative of the sequences disclosed in SEQ ID NOs 1, 3, or the coding region (nucleotides 1620-4873) of SEQ ID NO:5, as determined using oligonucleotide alignment algorithms (for example, but not limited to a BLAST or FASTA). Furthermore, oligonucleotides that associate with these sequences under conditions of high stringency, for example, but not to be limited to, hybridization to gel blots at about 65°C followed by wash conditions at 0.1X SSC, 65°C, are also considered *BNM3* genes. "*BNM*3 gene" also includes DNA molecules that comprises at least 23 contiguous nucleotides of SEQ ID NOs:1, 3 or 5, or at least 22 contiguous nucleotides within nucleotides 1-1619 of SEQ ID NO:5. A fragment of *BNM*3, that comprises at least 22 contiguous nucleotides may be used as a probe for the identification of nucleotides related to *BNM*3 regulatory, or coding, regions within an organism. Furthermore, molecules comprising at least 23 contiguous nucleotides of SEQ ID NOs:1, 3 or 5 and that encode a protein, or an active fragment thereof, that when present at a sufficient level within a plant cell renders the cell embryogenic, increases the regenerative capacity of the plant cell, or renders the cell embryogenic and increases the regenerative capacity of the plant cell are also considered to be *BNM*3 genes. Preferably, a *BNM*3 gene comprises from about 50 to about 1981 nucleotides of SEQ ID NOs: 1 or 3, or about 50 to about 4858 nucleotides from the coding region (1620-4858) of SEQ ID NO:5.

By "*BNM*3 regulatory region" it is meant the sequence of oligonucleotides from 1-1619 in SEQ ID NO:5, or a fragment, derivative, or mutation thereof. Furthermore, a *BNM*3 regulatory region also comprises a nucleotide sequence that associates with a sequence from 1-1619 of SEQ ID NO:5 under conditions of high stringency, for example, but not to be limited to, hybridization to gel blots at about 65°C followed by wash conditions at 0.1X SSC, 65°C, or that exhibits at least 70% homology or similarity, with a fragment or derivative of the sequences disclosed in nucleotides 1-1619 of SEQ ID NO:5, as determined using oligonucleotide alignment (for example, but not limited to a BLAST or FASTA search).

By "BNM3 protein" it is meant a protein, or a biologically active fragment thereof, that renders a plant cell embryogenic, increases the regenerative capacity of the

plant cell, or renders the cell embryogenic, increases the regenerative capacity of the plant cell, and that is encoded by a *BNM3* gene, as defined above. Preferably, a BNM3 protein comprises from about 30 to about 541 amino acids of the sequence disclosed in SEQ ID NO:2, or from about 30 to about 561 amino acids of the sequence disclosed in SEQ ID NO: 4. However, BNM3 protein may also be defined as a protein having at least 70% homology with either SEQ ID NO:2 or 4.

Search of the sequence databases indicated that the *BNM3* translation products contain two copies of an AP2 domain (Figure 3; see also SEQ ID NO: 2 for BNM3A, and SEQ ID NO: 4 for BNM3B). The AP2 domain was first identified in APETALA2, an arabidopsis protein that regulates meristem identity, floral organ specification, seedcoat development and floral homeotic gene expression (Jofuku *et al.*, 1994), but has since been identified in a wide range of proteins with diverse functions.

The AP2 domain is usually between 58 to 68 amino acids in length and contains a conserved central core of 18 amino acids, characterized by its ability to form an amphipathic α helix, a structure thought to mediate protein-protein interactions. The ability of a number of AP2 domain containing proteins to bind DNA, coupled with the presence of putative nuclear localization signals and acidic regions that may function as transcriptional activators suggests these proteins function as transcription factors.

Two phylogenetically distinct classes of AP2 domain proteins have been identified; proteins with a single AP2 domain (EREBP-like) and proteins with two AP2 domains (AP2-like; (Zhou, 1997)). The proteins encoded by the genes of this invention represent unique members of the latter class of proteins.

Accordingly, an aspect of the present invention provides for an isolated DNA molecule that comprises a sequence encoding a protein that contains two AP2 domains. The protein, when present at a sufficient level in a plant cell, renders the cell embryogenic, increases the regenerative capacity of the cell, or both renders the cell embryogenic and increases the regenerative capacity of the cell.

Analysis of *BNM3* expression during microspore-derived embryo development, seed development, or non-seed tissue development, using Northerns (Figure 6) indicated that the *BNM3* genes are preferentially expressed in embryogenic microspore cultures, microspore-derived embryos and seeds. *BNM3* transcripts were not detected in any of the non-seed tissues tested.

BNM3 mRNA is detected in microspore cultures induced to undergo embryogenesis, as well as in the subsequent globular, heart, torpedo and cotyledon stages of microspore-derived embryo development (e.g. Figure 6A). RNAs are also detected within developing seeds, 14 days after pollination (14 DAP), corresponding to the heart stage of embryo development. *BNM3* expression increases during the early (21 DAP) and mid-cotyledon (28 DAP) stages of embryo development and remains constant thereafter (Figure 6B).

Constitutive expression of *BNM3* resulted in the formation of somatic embryos on vegetative structures such as cotyledons, petioles, leaf blades and the shoot apical meristem of plants (Figure 7). In these experiments *BNM3* cDNAs were placed under the control of two separate constitutive promoter constructs, a modified sunflower *POLYUBIQUITIN* promoter construct, and a double enhanced *35S* promoter construct containing an AMV translational enhancer, however, it is to be understood that any suitable constitutive promoter may be used for this purpose. Such *BNM3*-derived ectopic embryos contain all of the organ systems and tissue layers found in the developing zygotic embryo in that these embryos are bipolar (Figure 7E), consist of an axis, a hypocotyl and radicle region, shoot and root meristems, and cotyledons. In addition, each organ system contained the characteristic radial arrangement of three specialized tissue layers (epidermis, ground parenchyma and provascular tissue) found in zygotic embryos. Continued expression of the *BNM3* gene within the developing ectopic embryo leads to a reiteration of the embryo-forming process, with the result that new embryos are continuously formed on the surface of pre-existing embryos (Figure 7E).

Constitutive expression of *BNM3* results in the increased ability of a plant to regenerate shoots *in vitro* in the presence of added growth regulators. Root explants from transgenic plants ectopicaly expressing *BNM3* show at least a 5-fold increase in shoot regeneration in the presence of hormones as compared to root explants obtained from wild-type plants (Figure 8A,B). Shoots also developed faster in the transgenic explants, compared to the wild-type. Wild-type leaf and hypocotyl explants initially responded by producing callus on the cut end of the petiole (Figure 8B) followed by callus formation along the length of the petiole. In contrast, explants from transgenic lines immediately produced new shoots (Figure 8B) or roots from the cut end of the petiole. Explants that initially produced roots eventually also produced shoots.

Transgenic explants, constitutively expressing *BNM3* were also able to regenerate in the absence of added growth regulators. These explants, when placed on media lacking growth regulators regenerated shoots either from the cut end of the leaf and hypocotyl explants or from the nodule-like structures of root explants (Figures 8C,D). In all cases regenerated shoots developed, rooted, flowered and set seed. Conversely, wild-type leaf and hypocotyl explants placed on medium lacking growth regulators occasionally produce callus or roots at the cut end of the leaf petiole, however no shoots form from these structures (Figure 8C,D).

It is also considered within the scope of the present invention, that expression of *BNM3* may be used to initiate a developmental cascade within a transformed plant or plant cell. This cascade may arise as a result of the stable integration of a DNA-based vector expressing BNM3 within a transformed plant, however, such a cascade may also arise as a result of transient expression of *BNM3*, and does not require the stable integration of the *BNM3*-based vector within a plant cell. These transient approaches may be useful for inducing somatic embryogenesis, gametophytically-derived embryogenesis, or increasing the regenerative capacity of a plant or plant cell.

Plants in which a *BNM3* gene is ectopically expressed exhibit advantageous qualities including:

- formation of asexually derived embryos;
- increased regenerative capacity of tissue explants;
- the ability of tissue explants to regenerate in the absence of added plant growth regulators; and
- the expression of seed components in non-seed organs in which *BNM3* is ectopically expressed.

Furthermore, plants that ectopically express at least one BNM3 gene can be used for the production of recombinant proteins using seed specific regulatory elements.

For the applications of BNM3 as described below, it will be advantageous to obtain a high level of the *BNM3* transcript and/or BNM3 protein in order to obtain plants in which the phenotype is highly penetrant. This may be obtained by using genetic elements such as introns, transcriptional enhancers or translational enhancers which are known to enhance gene or protein expression levels.

The BNM3 sequences of the present invention may be used for several applications including, but not limited to, the control of embryo processes, the control of regeneration processes, the use of regulatory sequences for targeted gene expression, the use of BNM3 sequences as selectable markers of transformed plants, or for embryogenic cells. These applications are disclosed in more detail below.

Use of *BNM3* Sequences to Control Embryogenic Processes

As described herein, *BNM3* genes play an important role in initiation and maintenance of embryo development. *BNM3* genes have been found in a wide range of members of the plant kingdom. Regulatory regions obtained from these genes may be used to control the transcription of *BNM3* or a derivative or fragment thereof, or any gene of interest, using methods known to one of skill in the art.

Ectopic expression of a *BNM3* gene is sufficient to induce recurrent formation of asexually derived embryos on the vegetative tissues of plants (see example 4). Depending

upon the promoter used, ectopic over-expression of *BNM3* genes may be used to produce somatic or gametophytic embryos. Somatic or gametophytic embryos may be obtained by expressing a *BNM3* gene under the control of a constitutive regulatory element, as is shown in Example 5, or may also be obtained by expressing a *BNM3* gene under the control of tissue specific or developmentally regulated elements, inducible elements derived from either plant or non-plant genes or through transient expression. In this respect, chemical induction systems (e.g. see Gatz and Lenk, 1998, which is incorporated by reference) or transient expression using methods which do not result in stable integration of the *BNM3* gene, or which make direct use of the BNM3 protein e.g. microprojectile bombardment of DNA or protein may also be employed.

Temporal and/or spatial restriction of *BNM3* expression using inducible, tissue specific or developmentally regulated elements, is preferred when recurrent embryogenesis is not a desirable trait. The regulatory elements used to restrict *BNM3* to a specific developmental stage or cell type will depend on the application. For example, regulatory elements that may be used to express *BNM3* for the production of microspore-derived embryos include, but are not limited to, those of the class I low molecular weight heat shock inducible gene, *GMHSP17.3B* (Zarsky *et al.*, 1995, which is incorporated by reference), or microspore/pollen expressed genes such as *NTM19* (Custers *et al.*, 1997 , EP 790,311, which are incorporated by reference), *BCP1* (Xu *et al.*, 1995, which is incorporated by reference), *LAT52* (Twell *et al.*, 1989, which is incorporated by reference), *BNM1* (Treacy et al 1997, which is incorporated by reference), and *APG* (Roberts *et al.*, 1993, which is incorporated by reference).

Examples of regulatory elements that may be used to express *BNM3* for the production of somatic embryos include, but are not limited to, those of genes activated by plant growth regulators which are routinely used to induce somatic embryogenesis in tissue culture. Specific examples, which are to be considered non-limiting, include the cytokinin inducible *IB6* and *CKI1* genes (Brandstatter and Kieber, 1998; Kakimoto, 1996, which are incorporated by reference) and the auxin inducible element, DR5 (Ulmasov *et*

al., 1997, which is incorporated by reference). However, it is to be understood that other regulatory elements may be included for the expression of *BNM3* in plants.

Furthermore, examples of gene regulatory elements suitable for directing expression of *BNM3* to obtain adventitious embryony include, but are not limited to, those obtained from the ovule and embryo expressed *SERK* gene (Schmidt et al, 1997 which is incorporated by reference), the ovule expressed *AGL11* gene (Roundsley *et al.*, 1995, which is incorporated by reference), the nucellus expressed *NUC1* gene (Doan *et al.*, 1996; WO 98/08961, which are incorporated by reference), or the inner integument-expressed genes, *FBP7* (Angenent *et al*, 1995, which is incorporated by reference) and *SC4* (US application 09/059,909, filed April 13, 1998, which is incorporated by reference) genes.

According to one aspect of the present invention there is provided a method for the efficient production of microspore-derived embryos in plants. This method involves:

i) transforming a plant of interest, for example, *Brassica napus* (using transformation techniques known to one of skill, for example, DeBlock *et al.*, 1989, Clough and Bent 1998, Vergunst et al. 1998, Klein et al 1987, which are incorporated herein by reference) with a vector construct, or isolated DNA, consisting of a *BNM3* gene under control of a suitable regulatory element, which may be constitutive, tissue specific, developmentally regulated, or inducible and, optionally, a marker gene for selection of transformants;
ii) selecting transformed plants;
iii) producing lines that ectopically overexpress the *BNM3* gene, or BNM3 protein;
iv) isolating microspores and pollen from the transgenic lines and culturing microspores and pollen to induce embryogenesis.

Embryogenesis can be induced by any suitable protocol, for example, which is not to be considered limiting, culturing microspore and pollen for about four days at from about 28° to about 35 °C, preferably at about 32°C, then transferring embryogenic cells or embryos to about 25 °C.

Using the above method, *Brassica napus* cultivars ectopically overexpressing *BNM3* show an increase in the percentage of embryogenic cells or embryos over that observed when microspores or pollen are prepared from wild-type plants that do not ectopically express *BNM3*.

Examples of regulatory elements that may be used to express *BNM3* for the production of microspore-derived embryos include, but are not limited to, those of the class I low molecular weight heat shock inducible gene, *GMHSP17.3B* (Zarsky *et al.*, 1995, which is incorporated by reference), or microspore/pollen expressed genes such as *NTM19* (Oldenhof *et al.*, 1996, EP 790,311, which are incorporated by reference), *BCP1* (Xu *et al.*, 1995, which is incorporated by reference), *LAT52* (Twell *et al.*, 1989, which is incorporated by reference), *BNM1* (Treacy et al 1997, which is incorporated by reference), and *APG* (Roberts *et al.*, 1993, which is incorporated by reference). Also useful are inducible regulatory elements, for example but not limited to, tetracycline-inducible promoter (Gatz 1997, which is incorporated by reference), steroid inducible promoter (Aoyama and Chua 1997, which is incorporated by reference) and ethanol-inducible promoter (Slater et al 1998, Caddick et al. 1998, which are incorporated by reference).

In a similar fashion, microspore-derived embryos may also be produced in plants by introducing into a plant of interest a BNM3 protein, (e.g. via biolistics ; Klein et al 1987) and selecting for plants that exhibit increased microspore embryogenesis.

This invention also provides a method for the efficient production of somatic embryos *in vitro*. This method involves:

i) transforming a plant, for example, *Arabidopsis* using transformation techniques known to one of skill (for example, but not limited to, DeBlock *et al.*, 1989, Clough and Bent 1998, Vergunst et al. 1998, which are incorporated by reference), or a plant cell may also be transiently transformed using methods

known to one of skill (for example, biolistics; Klein et al 1987) with a vector construct containing a *BNM3* gene under control of suitable regulatory element, which may be constitutive, inducible or developmentally regulated, and, optionally, a marker gene for selection of transformants is transformed to several arabidopsis.

ii) selecting transformed plants, and

iii) culturing the desired explant from the selected transformed plants, for example, but not limited to, root, leaf or seedlings *in vitro*, in media with or without appropriate growth regulators, for example, but not limited to 2,4-D (e.g. Mordhorst *et al.*, 1998) to produce direct embryogenesis or embryogenic callus; and

iv) transferring embryos, non-embryogenic callus, or both embryos and non-embryogenic callus to appropriate media for the production of embryos, plantlets, or both embyos or plantlets.

For example, when the results of the above method are compared with the production of somatic embryos *in vitro* using a number of *Arabidopsis* ecotypes, directed embryogenesis or embryogenic callus is initiated at a higher frequency from transgenic lines ectopically over-expressing *BNM3* than in wild-type controls.

Examples of regulatory elements that may be used to express *BNM3* for the production of somatic embryos include, but are not limited to, those of genes activated by plant growth regulators which are routinely used to induce somatic embryogenesis in tissue culture. Specific examples, which are to be considered non-limiting, cytokinin inducible *IB6* and *CKI1* genes (Brandstatter and Kieber, 1998; Kakimoto, 1996, which are incorporated by reference) and the auxin inducible element, DR5 (Ulmasov *et al.*, 1997, which is incorporated by reference). Also useful are inducible regulatory elements, for example but not limited to, a teracycline-inducible promoter (Gatz 1997, which is incorporated by reference), a steroid inducible promoter (Aoyama and Chua 1997, which is incorporated by reference), and an ethanol-inducible promoter (Slater et al 1998, Caddick et al. 1998, which are incorporated by reference).

Ectopic initiation of embryo development is one of the key steps in apomixis. As shown in Example 4, ectopic expression of a *BNM3* gene is sufficient to initiate embryo formation in otherwise non-embryo-forming tissue. A *BNM3* gene may therefore be used to initiate adventitious embryony or parthenogenesis of a reduced or unreduced embryo sac cell by expression of the gene in the sporophytic or gametophytic tissues of the developing ovule.

Adventitious embryony is achieved by expressing *BNM3* in sporophytic ovule tissues such as the nucellus, the inner integuments or other tissues lying adjacent to or in proximity to the developing embryo sac. This method involves:

i) transforming a desired plant (see above methods) with a vector construct consisting of a *BNM3* gene under control of suitable regulatory element, which may be constitutive, inducible or developmentally regulated, and, optionally, a marker gene for selection of transformants, using methods known within the art;
ii) selecting transformed plants;
iii) emasculating the transformed plant;
iv) pollinating the transformed plants with pollen carrying one or more dominant selectable markers, for example GUS or kanamycin resistance; and
v) assaying for production of clonal offspring.

When the results of the above method are compared with the pollination of a wild-type arabidopsis plant with pollen carrying the dominant selectable marker, all F1 embryos resulting from this cross inherit the dominant marker while embryos derived from plants ectopically over expressing the BNM3 gene or protein are clonally derived via sexual embryo formation and do not inherit the dominant selectable marker.

Specific examples of gene regulatory elements suitable for directing expression of *BNM3* to obtain adventitious embryony, diplospory or haploid parthenogenesis of embryo sac components include the ovule expressed SERK gene (Schmidt et al. 1997, which is incorporated by reference), the meiosis expressed *AtDMC1* gene, (Klimyuk and Jones, 1997; WO 98/28431, which are incorporated by reference), the ovule expressed

AGL11 gene (Roundsley *et al.*, 1995, which is incorporated by reference), the nucellus expressed *NUC1* gene (Doan *et al.*, 1996; WO 98/08961, which are incorporated by reference), and the inner integument-expressed genes, *FBP7* (Angenent *et al*, 1995, which is incorporated by reference) and *SC4* (US application 09/059,909, filed April 13, 1998, which is incorporated by reference) genes. Furthermore, inducible systems, for example but not limited to, tetracycline-inducible promoter (Gatz 1997, which is incorporated by reference), steroid inducible promoter (Aoyama and Chua 1997, which is incorporated by reference), ethanol-inducible promoter (Slater et al 1998, Caddick et al. 1998, which are incorporated by reference) may also be used. Parthenogenesis from cells of the embryo sac requires a regulatory element that is active in one or more cells of the female gametophyte or their precursors. Fertilization of the meiotically-derived polar nuclei is desirable when the development of seed is dependent on the presence of endosperm.

Use of *BNM3* Sequences to Control Regeneration Processes

Plants ectopically over-expressing the *BNM3* genes exhibit increased regenerative capacity and the ability to regenerate whole plants in the absence of added growth regulators (see example 5). *BNM3* gene expression may therefore be used to enhance or induce the regeneration capacity of plant tissues *in vivo* or *in vitro*. The regulatory elements used to express *BNM3* will depend, in part, on the target tissue used for regeneration. Regeneration of plant tissues may be obtained by expressing a *BNM3* gene under the control of a constitutive regulatory element, for example, but not limited to, 35S, or by expressing a *BNM3* gene under the control of tissue specific or developmentally regulated elements, inducible elements derived from either plant or non-plant genes (e.g. Gatz and Lenk, 1998, which is incorporated by reference), or through transient expression methods which do not result in stable integration of the *BNM3* gene or which make direct use of the BNM3 protein (e.g. microprojectile bombardment of DNA or protein). Chemical induction systems (see Gatz and Lenk, 1998) or regulatory elements of genes that respond to plant growth regulators used to induce regeneration, such as, for example, cytokinin (Brandstatter

and Kieber, 1998; Kakimoto, 1996) or auxin (Ulmasov et al., 1997), or genes expressed at the wound site of tissue explants (Xu *et al.*, 1993) may be used.

A further application is the use of a *BNM3* gene as a selectable marker for the recovery of transgenic plants. As an example of this application which is not to be considered limiting in any manner, roots of a seedling, for example, an *Arabidopsis* ecotype C24 seedling, are cocultivated with a single *Agrobacterium tumefaciens* strain (per Vergunst *et al*, 1998; except that all steps are carried out in the absence of added growth regulators) containing two binary constructs:

- a first binary vector carries a reporter gene fusion, for example, but not limited to, *35S:GUS*;
- a second binary vector contains a *BNM3* gene under control of suitable regulatory element.

BNM3 gene expression is activated upon integration of the above construct into the arabidopsis genome and transgenic plants are selected on the basis of their ability to regenerate under conditions in which wild-type explants are unable to regenerate, for example, but not limited to, the absence of growth regulators. In many instances the T-DNA carrying the *BNM3* gene and the T-DNA carrying the gene of interest will integrate at unlinked loci. The T-DNA containing the introduced *BNM3* sequence,and it's associated increased regenerative capacity phenotype, may therefore be removed in the progeny plants by simple segregation (Daley et al. 1998). However, as will be apparent to one of skill in the art, other methods such as transient expression, which do not result in stable integration of the BNM3 gene or which make direct use of the BNM3 protein, may also be employed.

Use of *BNM3* Sequences to Target Gene Expression to the Embryo

Since *BNM3* genes are preferentially expressed in developing embryos (see example 3), a further application of this invention is the use of *BNM3* regulatory regions to target expression of at least one heterologous gene of interest to the developing embryo for any purpose, for example, but not limited to, altering embryo

and seed traits such as seed viability or size, composition of constituents of the seed, disease resistance, or the production of high value products such as vaccines antibodies, biopharmaceuticals or other specialty chemicals.

Use of *BNM3* Expression as a Marker for Embryogenic Cells

As shown in Examples 3 and 4, *BNM3* gene expression is detected during the earliest phase of plant embryogenesis and is itself sufficient to activate signal transduction cascades leading to embryo development. *BNM3* gene expression is therefore a specific marker for the entry of a plant cell into the embryogenic pathway.

BNM3 expression is associated with embryo-forming cell divisions *in vitro* and *in vivo* and as such can be used to define culture conditions that alter the embryo-forming capacity of a tissue *in vitro*. Cells with embryogenic capacity or cells that undergo only a limited number of embryo-forming divisions are difficult to identify in the absence of structures that morphologically resemble embryos. However, these cells may be identified on the basis of *BNM3* expression. In this application, a vector containing the *BNM3* regulatory region, fused to a reporter gene, for example, but limited to, *GUS* (Jefferson *et al.*, 1987), *Luciferase* (Ow *et al.*, 1987) or *GFP* (Haselhoff and Amos, 1995) is transformed to a plant of interest. Homozygous transgenic lines exhibiting high levels of reporter gene expression in the embryo are cultured under *in vitro* conditions. Embryogenic cells, as well as culture conditions which facilitate or enhance the formation of embryogenic cells are identified on the basis of reporter gene expression within the cultured tissue.

A related application is the use of the *BNM3* gene as a marker in apomictic species for the identification of individual cells that are in the process of forming asexually-derived embryos. In this application, cells entering the autonomous embryo pathway are identified by mRNA *in situ* hybridization using a RNA probe derived from a *BNM3* gene sequence, by immunocytochemistry using a antibody directed against a BNM3 protein, by transforming plants with a DNA construct containing a

gene fusion between *BNM3* regulatory regions and a reporter gene, or by any similar technique known to those skilled in the art.

Identification of Signal Transduction Components

Signal transduction components which activate or are activated by *BNM3* gene expression can be elucidated by identifying proteins and DNA sequences that interact with a *BNM3* gene and its protein product. These signal transduction components may be identified using techniques known to a person skilled in the art, including for example, but not limited to:

- yeast one hybrid screens for the isolation of proteins that bind to the *BNM3* regulatory regions to influence *BNM3* gene expression;
- genetic selection in yeast to identify genes that are direct targets of BNM3 binding;
- DNA arrays or proteomics to identify genes which are activated in a BNM3 signal transduction cascade; and
- yeast two hybrid screens to identify proteins that interact with BNM3 to influence expression of downstream target genes.

Techniques for the analysis of the signal transduction components and signalling components are well known (see for example, Meijer *et al.* (1998), Lipshutz *et al.* (1999), and Anderson and Anderson (1998)).

Plants over-expressing the *BNM3* gene under control of a strong constitutive regulatory element such as, for example, but not limited to, the Cauliflower Mosaic Virus *35S* promoter exhibit ectopic embryo formation, enhanced regeneration via organogenesis or a combination thereof (Examples 4 and 5). The ability of *BNM3* ectopic over-expression to induce both embryo formation and enhance regeneration processes can be used to identify mutants altered in their embryo-forming or regenerative capacity. In this application a vector construct consisting of a *BNM3* protein coding region under control of a regulatory element that is sufficient to promote either ectopic embryo formation or enhanced regeneration phenotype is made

and introduced into a plant of interest. Homozygous transgenic lines exhibiting a high penetrance of ectopic embryo formation, enhanced regeneration phenotype, or a combination thereof are identified. These lines are mutagenized by any available technique well known to the person skilled in the art, but which may include EMS mutagenesis, fast neutron mutagenesis, transposon mutagenesis or T-DNA mutagenesis. Mutagenized plants are then screened for alterations in the ectopic embryo formation or regeneration phenotype. These alterations include, for example, but not limited to, elimination or enhancement of the ability to promote ectopic asexual embryo formation or to regenerate in the absence of added growth regulators.

Heterologous Protein Expression System

Genetic control of the signal transduction pathway leading to embryogenesis and organogenesis in non-seed organs of transgenic plants may be activated by ectopic expression of a *BNM3* gene. Expression of a *BNM3* gene in association with a heterologous promoter can be used to produce altered seed components including for example, proteins, oils and other metabolites. Biotransformation of desired organs may also include altering the nutritive value of, for example leaves of forage crops, or it may be used to create alternative uses for crops. The use of promoters that are induced by the signal transduction cascade initiated by expression of *BNM3* can be used to express high-valued recombinant proteins in organs other than seeds. An example of one such promoter is the napin promoter, obtained from the 2S seed storage protein napin. The production of proteins initiated from a *BNM3*-induced cascade, may be achieved within organs exhibiting greater biomass than seeds. Therefore, this technology may be used to create alternatives for plants as crops.

Accordingly, the present invention further relates to a binary system in which the BNM3 protein binds directly or indirectly to an embryo-expressed regulatory sequence (target sequence) and activate transcription of a chimeric gene construct in any plant cell, tissue or organ. Therefore, BNM3 may be used to directly or indirectly activate transcription of a chimeric gene construct. This approach involves BNM3

interacting either directly with at least one target sequence from an embryo-expressed gene, or indirectly by initiating an embryogenic signal cascade that activates a transcription factor that in turn binds to and activates transcription from at least one target sequence. This binary system may be used for the expression of proteins in somatic tissues with the properties of expression in seeds.

In this application transgenic plants containing the *BNM3* gene under control of a constitutive regulatory element, for example, but not limited to the *35S* promoter (*35S:BNM3*) are created to produce a BNM3 activator line. *BNM3* expression may be demonstrated in a wide range of tissues in the BNM3 activator lines by RNA gel blot analysis. Stable homozygous activator lines with high levels of *BNM3* expression are identified. Somatic tissues over-expressing *BNM3* may be examined for expression of other embryo-expressed genes, such as arabin (Guerche et al., 1990), cruciferin (Pang *et al.*, 1988) or oleosin, or for morphological properties that are normally characteristic of seeds, such as the presence of lipid or protein bodies.

Transgenic plants of the same species to that used to generate the BNM3 activator lines described above are also created which contain an embryo-expressed promoter fused to a gene of interest, to produce a gene of interest line. In order to help describe this embodiment, the gene of interest line expresses a reporter gene, such as *GUS* , and examples, which are not to be considered limiting, of such lines include *Brassica napus* 2S albumin seed storage protein gene, *BnpNAP1:GUS* fusion (Baszczynski *et al.*, 1994) or a *SERK:GUS* fusion (Schmidt *et al.*, 1997; a non-seed expressed reporter construct such as *BNM1:GUS* (Treacy *et al.*, 1997) may be used as a negative control). The fidelity of expression of the gene of interest in the specific organs and tissues of these gene of interest lines is demonstrated for each construct. Stable homozygous lines with high levels of expression of the gene of interest expression are created.

Transgenic lines containing BNM3 activator lines and gene of interest lines are crossed and the progeny seeds collected. *BNM3* gene expression, and in this example,

GUS activity, expression of other embryo-expressed genes, as well as the morphological characteristics of transformed tissues, are examined. *BNM3* expression in non-seed tissues typically activates both embryo development and expression of the gene of interest (e.g. GUS), however, activation of the expression of the gene of interest in the absence of morphologically discernible embryos may also be observed. Expression of the gene of interest, in the absence of morphologically discernible embryos provides initial evidence for direct interaction of BNM3 with the target sequence.

Direct interaction of BNM3 with a target sequence may also be demonstrated using transient expression of BNM3 in plant protoplasts, along with the transient co-expression of an embryo-expressed promoter fused to a gene of interest (i.e. a gene of interest construct). *35S:BNM3* DNA and the gene of interest construct are introduced into protoplasts derived from non-seed cells, such as leaf mesophyll cells by electroporation. The expression of the gene of interest is examined after several hours to confirm activation of the target sequence. Direct interaction of BNM3 with the target sequence may further be demonstrated by co-introducing the target sequence alone as competitor DNA.

In order to determine if tissues from different plant species may be transactivated by BNM3, *35S:BNM3* DNA and a reporter gene (for example, but not limited to *GUS*) construct may be introduced by microprojectile bombardment into somatic tissues of a plant. If BNM3 interacts directly with a target sequence then expression of the report er gene should coincide with transient expression of BNM3 in all species and tissues.

Direct evidence for BNM3-target sequence interaction may also be obtained by isolation of BNM3 protein expressed in bacteria, insect or yeast. *BNM3* is expressed in bacteria, insect, or yeast using commercially available expression systems and isolated to purity. Gel mobility shift assays (Gustavson *et al.*, 1991) are performed using a BNM3-target sequence, for example an embryo-expressed target

sequence, to demonstrate direct binding of BNM3 to the BNM3-target sequence. Footprint analyses may also be performed to locate the region of BNM3 binding. Fragments of target sequences that bind BNM3 may then be subcloned and used as competitors for BNM3 binding in transient assays described above.

The present invention will be further illustrated in the following examples. However it is to be understood that these examples are for illustrative purposes only, and should not be used to limit the scope of the present invention in any manner.

Examples

General methods: *Microspore Embryo Culture*

Brassica napus c.v. Topas was used as the source of all plant material for microspore embryo culture. Donor plants for microspore culture were grown in a growth cabinet at 20 °C /15 °C (day/night) with a 16 h photoperiod (400 µE/m/s) provided by VHO cool white fluorescent lamps (165W, Sylvania) and incandescent bulbs (40W, Duro-test). Four weeks after germination the plants were transferred to growth cabinets under the same light conditions, but set at 10 °C /5 °C (day/night). Microspores and pollen were isolated and cultured as described in Keller *et al.* (1987), except that after 21 days in culture, cotyledon stage embryos were transferred to a maturation medium consisting of 1/2X NLN salts, 1% sucrose, 0.35 M mannitol and 5 µM ABA. Uninduced cultures (microspores and pollen continuing gametophytic development) and heat-stressed, non-embryogenic cultures (used for construction of the subtracted probe), were cultured from the same starting material as was used for the initiation of embryogenic cultures. Uninduced samples were obtained by culturing microspores and pollen for four days at 25 °C. Heat-stressed, non-embryogenic samples were obtained by culturing microspores and pollen for one day 25 °C, followed by three days 32 °C.

Samples of microspore and pollen cultured for less than 10 days were collected by centrifugation. Older samples containing globular, heart, torpedo and cotyledon stage microspore-derived embryos were collected by filtration through nylon meshes of various pore sizes as described in Ouellet *et al.* (1992). All other plant tissues were collected from greenhouse grown material. Seed material was obtained by hand pollinating flowers on the day of anthesis and collecting developing seeds on various days after pollination (DAP).

Nucleic Acid Isolation and Analysis

Total RNA was isolated using either a cesium chloride/guanidinium isothiocyanate procedure (Ouellet, 1992) or TRIZOL reagent (Gibco-BRL). RNA gel blot analysis was carried out by separation of 5 to 20 µg of total RNA per lane through 1.5% agarose gels containing 0.62 M formaldehyde, essentially as in Sambrook *et al.* (1989), followed by capillary transfer to Hybond-N nylon membranes (Amersham). Poly(A)$^+$ RNA was isolated from total RNA by oligo (dT)-cellulose chromatography (Sambrook,1989).

Genomic DNA was isolated from leaf tissue as described in Fobert *et al.* (1991) and digested with the specified restriction enzymes using standard procedures (Sambrook,1989). DNA gel blot analysis was carried out by electrophoresis of 10 µg DNA through 0.8% agarose gels followed by capillary transfer to Hybond-N membranes.

The partial 1.2 kb *BNM3A* cDNA insert was used as a probe for DNA and RNA gel blots. Hybridization to gel blots was carried out at 65 °C according to the Hybond-N protocol. The final wash conditions were 0.1X SSC, 65 °.

Subtractive Probe Construction and cDNA Library Screening

Poly (A) mRNA was isolated from late uninucleate microspores and early binucleate pollen that had been cultured for four days at 32 °C in order to induce embryogenesis (embryogenic sample) and used to synthesize first strand cDNA (Riboclone cDNA kit; Promega). The cDNA was then hybridized to a five-fold excess (by weight) of poly $(A)^+$ RNA from late uninucleate microspores and early binucleate pollen that had been cultured for one day at 25 °C, followed by three days at 32 °C to inactivate embryogenesis (non-embryogenic sample: Pechan *et al.*, 1991). The subtractive hybridization was performed essentially as described in Sambrook *et al.* (1989). The single-stranded cDNA recovered after subtraction was labelled with [α-^{32}P] dCTP using a random primers kit (BRL) and used as the subtracted probe for screening a Lambda phage cDNA library constructed from the same embryogenic sample described above (Boutilier, 1994). Triplicate nylon filter lifts (Hybond-N) from approximately 1.5 x 10^5 plaque-forming units of the library were screened with the subtracted probe, with a random primers-labelled first strand non-embryogenic probe and with a random primers-labelled napin seed storage protein cDNA probe (pN2; (Crouch, 1983). Napin mRNAs are prevalent in the embryogenic microspore library (Boutilier, 1994) and therefore plaques hybridizing to the napin probe were removed from the subsequent screening steps. Plaques hybridizing to the subtracted probe, but not to the non-embryogenic or napin probes, were selected and subjected to two subsequent rounds of differential screening using both the subtracted and non-embryogenic cDNA probes. DNA from selected Lambda clones was isolated (Sambrook, 1989), partially digested with *Eco* RI and *Xba* I and subcloned into pGEM-4Z (Promega).

Seven cDNAs comprising 6 unique genes, one of which comprised a truncated BNM3A cDNA, were identified. Two distinct, full length BNM3 cDNA clones (BNM3A and BNM3B) were subsequently obtained by stringent screening of circa 2.5 x 10^5 plaque-forming units of a cDNA library (UniZAPII cDNA synthesis kit, Stratagene) constructed with mRNA from 10 day old globular to heart-stage microspore-derived embryos of *B. napus* c.v. Topas. The BNM3 cDNA inserts were rescued by *in vivo* excision into Blueskript SK(-) (Stratagene).

Isolation of Genomic DNA sequences

The Universal Genome Walker Kit (Clonetech) was used to isolate genomic DNA fragments lying upstream of the *BNM3* ATG start codon. Pools of uncloned, adaptor-ligated *Brassica napus* cv Topas genomic DNA fragments were constructed and used to isolate BNM3 genomic sequences by nested PCR. The primary PCR made use of the outer adaptor primer (AP1) supplied by the manufacturer and a BNM3 specific primer with the sequence:

5'-GAGGCAGCGGTCGGATCGTAACAGTACTCT-3' (SEQ ID NO:6).

The nested PCR made use of the nested adaptor primer (AP2) supplied by the manufacturer and a BNM3 specific primer with the sequence:

5'-CATAAGGAGAGAGAGAAAAGCCTAACCAGT - 3' (SEQ ID NO:7).

The primary PCR mixture was then diluted 1:50 and used as template for nested PCR. Both the primary and nested PCRs were performed as recommended by the manufacturer. The nested PCR products were cloned into the pGEMT-Easy vector (Promega) and sequenced. PCR products corresponding to the 5' untranslated genomic regions of both *BNM3A* and *BNM3B* cDNAs were identified.

The genomic DNA sequence spanning the *BNM3*A ATG translational start and TAG translational stop codons was isolated by PCR from *B. napus* cv Topas genomic DNA using *Pfu* polymerase (Stratagene) and the following primer combination:

5'-ACCAAGAACTCGTTAGATC-3' (SEQ ID NO:8); and

5'-AACGCATATAACTAAAGATC-3' (SEQ ID NO:9).

The primers were used under standard PCR conditions. The PCR products were cloned into the pGEMT-Easy vector and sequenced.

Plasmid Construction for Plant Transformation

The construction of a plasmid vectors containing the *BNM3* cDNAs under control of either a *POLYUBIQUITIN* or Cauliflower Mosaic virus 35S promoter are

described below. The plasmid pRAP2TUBI contains a modified *Helianthus annus POLYUBIQUITIN* promoter (Binet *et al.*, 1991) in the plasmid pRAP2T. The plasmid pRAP2T consists of the pUCAP plasmid (van Engelen *et al.*, 1995) and a nopaline synthase (nos) terminator inserted into the *Sac* 1 and *Eco* R1 restriction sites. A PCR fragment of the *POLYUBIQUITIN UbB1* promoter comprising the 5' end of the promoter to 7 bp from the 3' end of the first exon was amplified from the vector using an M13 reverse primer and the UBIQ-3' primer:

5'-CCATGGATCCAGAGACGAAGCGAAAC-3' (SEQ ID NO:10)

which includes introduced *Nco* I and *Bam* HI restriction sites. The *POLYUBIQUITIN* promoter fragment was digested with *Pst* I and *Bam* HI, gel purified and ligated into the *Pst* I and *Bam* HI sites of pRAP2T, creating the vector pRAP2TUBIHa. The full-length *BNM3B* cDNA was digested with *Eco* RI and *Xho* I restriction enzymes, blunted with Klenow enzyme, gel purified and ligated into the *Sma* I site of pRAP2TUBI making the plasmid pKB1S. An *Asc I/Pac* I DNA restriction fragment containing the modified *POLYUBIQUITIN* promoter, the *BNM3B* cDNA and the nos terminator was gel purified, and ligated to the *Asc I/Pac* I digested binary vector pBINPLUS (van Engelen *et al.*, 1995), creating the plasmid pKBBIN1S.

The construction of a vector containing the *BNM3A* cDNA under control of a double enhanced 35S promoter and AMV translational enhancer was as follows. A *Hind* III/*Xba* I DNA restriction fragment containing the double 35S promoter and the AMV translational enhancer from plasmid pBI525 (Datla et al., 1993) was ligated to *Hind* III/*Xba* I digested pRAP2T, creating the plasmid pRAP2T35S. An *Nco* I site was introduced into the *BNM3A* cDNA clone by site directed mutagenesis. The sequence of the BNM3ANCO1 primer used for mutagenesis is:

5'-ACTCCATGGATAATAACTGGTTAGGC-3' (SEQ ID NO:11).

A second primer, BNM3AHINDIII:

5' - AAATTCTCAAGCTTTGGTCCATCTTG-3' (SEQ ID NO12)

was used together with the BNM3ANCO1 primer to amplify a 305 bp fragment of the *BNM3A* cDNA. This PCR fragment was digested with *Nco* I and *Hind* III and ligated to *Nco I/Kpn*I cut pRAP2T35S and a *Hind* III/*Kpn* I fragment containing the region of

the *BNM3A* cDNA downstream of the *Hind* III site, creating the vector p35S:BNM3. p35S:BNM3 was digested *Asc* I and *Pac* I restriction enzymes and the fragment containing the double 35S promoter, the AMV translational enhancer, the BNM3A cDNA and the nos terminator was gel purified and ligated to the *Asc I/Pac* I digested binary vector pBINPLUS, creating the plasmid p35S:BNM3BIN.

Both the pKBBIN1S and p35S:BNM3BIN plasmids were transferred to *Agrobacterium tumefaciens* C58C1 strain carrying the disarmed Ti plasmid pMP90 and used in transformation experiments.

Plant Transformation

Arabidopsis thaliana ecotype C24 was used as the recipient in transformation experiments. Plants were transformed using either the floral dip method described in Clough and Bent (1998) or the root transformation method described in Vergunst *et al.* (1998).

Transgenic *Brassica napus* c.v."Topas" plants were produced by *Agrobacterium tumaciens*-mediated transformation of microspore-derived embryos. Microspore-derived embryos were cultured for 5 weeks at a density of approximately 1000 embryos per ml. Overnight cultures of *Agrobacterium* were diluted 100 times in B5 medium containing 9% sucrose. Embryos were co-cultivated with the diluted bacteria for 48 hours at 24□C in darkness, with slow shaking. The embryos were then transfered to NLN13 medium supplemented with 350 mg/L cefotaxim and 200 mg/L vancomycin for at least two weeks in darkness at 25 °C.

Embryos were germinated in weak light at 25 °C for about 2 weeks on solid B5 medium supplimented with 2% sucrose, cefotaxim (200 mg/L) and vancomycin (100 mg/L). Well developed hypocotyls from germinated embryos were isolated and transferred to fresh germination medium supplimented with 100 mg/L kanamycin. After two weeks on this medium, explants were subcultured to a similar medium

supplemented with kanamycin (25 mg/L). Green, putative transgenic, secondary embryos become visible after one month of selection.

Microscopy

All plant material was fixed overnight at 4 °C in 0.1 M phosphate buffer pH 7.0 containing 4% paraformaldehyde. Samples were washed in 0.1 M phosphate buffer and then dehydrated in a graded ethanol series to 100% ethanol. Samples for scanning electron microscopy were critical point dried in liquid CO_2 (Balzers CPD020), and mounted on SEM stubs using conductive carbon glue. Samples were coated with 30 nm palladium/gold using a Polaron E5100 sputter coater. Samples were observed in a JEOL JSM 5200 scanning electron microscope with an acceleration voltage of 15 kV. Digital images were obtained using Orion Framegrabber. Samples for light microscopy were embedded in Technovit 7100 (Kulzer). Sections were stained for 10 seconds in 1% Toluidine blue in 1% sodiumtetraborate, rinsed with water and mounted in Euparal. Digital images were recorded using a Sony 3 CCD camera.

Regeneration Experiments

Wild-type and transgenic arabidopsis seeds were surface sterilized, plated on 1/2 MS media containing 20% sucrose (½MS-20) and grown at 21□C with the plates inclined at a 60° angle. Eight wild-type seedlings and eight seedlings from each of seven independent transgenic lines were harvested 10 days after germination and separated into root, hypocotyl and leaf explants. This material was then divided into two batches. Half of the explants were continuously cultured on B5 media containing 20% glucose (B5-20). Explants were transferred to fresh B5-20 media every two weeks. The remaining explants were cultured on B5-20 containing plant growth regulators in order to induce shoot regeneration (Vergunst *et al.*, 1998). These explants were first placed on callus inducing media (CIM; high auxin to cytokinin ratio) for two days and then transferred to shoot inducing media (SIM; high cytokinin

to auxin ratio) for the remainder of the culture period. Explants were transferred to fresh SIM media every two weeks.

Example 1: Isolation and Characterization of the BNM3 Genes from Brassica napus

A subtractive screening approach was used to isolate genes preferentially expressed during the induction of *Brassica napus* c.v. Topas microspore embryogenesis (Figure 1). Two types of microspore cultures were used in the construction of a subtracted probe: embryogenic and non-embryogenic. Embryogenic cultures were obtained by subjecting late uninucleate microspores and early binucleate pollen to a 4 day, 32 °C heat stress treatment. The non-embryogenic sample was obtained by culturing the same starting population of late uninucleate microspores and early binucleate pollen for 1 day at 25 °C followed by 3 days at 32 °C (Pechan *et al.*, 1991). Poly(A) mRNA was isolated from the embryogenic sample and used to synthesize first strand cDNA. The cDNA was then hybridized to an excess of poly(A)$^+$ RNA isolated from a non-embryogenic microspore/pollen sample. The non-hybridizing, single stranded cDNA, enriched for sequences present in the embryogenic sample, but absent or present at a much lower level in the non-embryogenic sample, was recovered, radioactively labelled and used as a subtracted probe for screening a cDNA library derived from the embryogenic sample described above. Plaques hybridizing to the subtracted probe, but not to a probe derived from the non-embryogenic sample, were selected and subjected to two subsequent rounds of differential screening. Seven independent cDNA clones, comprising six unique DNA sequences were found to be differentially expressed between the embryogenic and non-embryogenic samples. One of these clones, 42A1, later renamed *BNM3A* (for *Brassica napus* microspore embryo), was further characterized.

Example 2: The BNM3 genes encode new members of the AP2 domain class of transcriptional activators

A single *BNM3* cDNA clone, *BNM3A*, was isolated after screening an embryogenic microspore cDNA library with a subtracted probe enriched for genes expressed in embryogenic microspores and pollen. The discrepancy between the size of the cDNA clone (1.2 kb) and the size of the transcript detected on RNA gel blots (2.2 kb) indicated that this clone did not represent a full-length cDNA. Two longer cDNA clones, corresponding to the full length cDNA of the clone originally isolated, *BNM3A* (SEQ ID NO. 1), and a new clone, *BNM3B* (SEQ ID NO. 3), were isolated from a 10 day old *Brassica napus* microspore embryo cDNA library. The alignment of the DNA sequence of these clones is shown in Figure 2. The two *BNM3* cDNA clones are 2011 and 1992 nt in length, and are 97% similar at the nucleotide level, differing only slightly in the length and sequence of their 5' and 3' untranslated regions. Both cDNAs potentially encode 579 amino acid polypeptides (predicted molecular mass of 63.9 kDa, pI of 5.7) that are 97% similar at the amino acid level (Figure 3).

The genomic complexity of the *BNM3* genes was determined by hybridization of the *BNM3* cDNAs to gel blots containing *B. napus* genomic DNA (Figure 4). The *BNM3* cDNAs hybridize to two DNA fragments under high stringency conditions. The two hybridizing fragments represent the two *BNM3* genes, *BNM3A* and *BNM3B*. *B. napus* is an amphidiploid species derived from the hybridization of the diploid *B. rapa* and *B. oleracea* genomes, thus the two *BNM3* sequences are likely derived from a single copy locus in each of the parental diploid progenitors.

Search of the sequence databases indicated that the *BNM3* translation products contain two copies of an AP2 domain (Figure 3). The AP2 domain was first identified in APETALA2 (AP2), an arabidopsis protein that regulates meristem identity, floral organ specification, seedcoat development and floral homeotic gene expression (Jofuku *et al.*, 1994; WO 98/07842), and has since been identified in a wide range of proteins with diverse functions. These functions range from the activation of genes involved in stress (Zhou, 1997; Stockinger, 1997) and ethylene response (Ohme-Takagi, 1995) to the regulation of leaf, floral and ovule development (Moose, 1996; Jofuku, 1994; Elliot, 1996; Klucher, 1996). The AP2 domain is a 56-68 amino acid

repeated motif containing at least two conserved regions: a highly basic YRG element, containing a conserved YRG amino acid motif and the RAYD element. The RAYD element contains a conserved central core of 18 amino acids that is predicted to form an amphipathic α-helix, a structure that is thought to mediate protein-protein interactions. The ability of a number of AP2 domain containing proteins to bind DNA, coupled with the presence of putative nuclear localization signals and acidic regions that may function as transcriptional activators suggests these proteins function as transcription factors.

Two phylogenetically distinct classes of AP2 domain proteins, consisting of either one AP2 domain (EREBP-like) or two AP2 domains connected by a linker region (AP2-like), have been identified (Zhou, 1997). BNM3 belongs to the latter class. Search of the databases with the region corresponding to the two AP2 domains and linker region of BNM3 reveals that BNM3 is most similar to the arabidopsis AINTEGUMENTA (ANT; Elliot, 1996;Klucher, 1996) and the *Zea mays* ZMMHCF1 AP2 domain containing protein. (ZM; Daniell, 1996) Figure 5 shows an alignment of the two AP2 domains of BNM3 with those of other proteins that contain two AP2 domains. BNM3 shares 85% amino acid sequence similarity with ANT and 88% with ZMMHCF1 in this region, but only 66% amino acid similarity with AP2 and GLOSSY15 in this region. A 10 amino acid insertion in the first AP2 domain of the and BNM3 proteins further distinguishes these three proteins from other AP2 domain containing proteins (Elliot, 1996). The BNM3, AINTEGUMENTA and ZMMHCF1 proteins also share a small hydrophobic amino acid motif, LG/SFSLS, in their amino terminal regions, but otherwise show no significant similarity in their DNA or amino acid sequences outside of the AP2 domains and linker. These results indicate that the BNM3 sequences encode unique members of the AP2 domain family of proteins.

A pairwise alignment of BNM3B cDNA and amino acid, sequences with ANT or ZMMHCF-1 sequences indicated that for the BNM3B nucleotide sequence:

- there is a 56% identity with ANT cDNA (over the 1905 nucleotides of ANT) and a 58% identity with ZMMHCF1 cDNA (over the 1773 nucleotide sequence of ZM);

and for the BNM3B amino acid sequence:

- there is a 41% identity of the BNM3B protein with ANT protein (over the 555 amino acid sequence of ANT), and a 46% identity with ZMMHCF1 protein (over 485 amino acid sequence of ZM).

Example 3: The BNM3 genes are preferentially expressed in developing embryos

RNA gel blot analysis (Figure 6) was used to determine the pattern of *BNM3* gene expression during microspore-derived embryo development, seed development, and in non-seed tissues. Both analyses indicate that the *BNM3* genes are preferentially expressed in developing embryos.

RNA gel blot analysis indicates that *BNM3* mRNAs are detected in microspore cultures induced to undergo embryogenesis, as well as in the subsequent globular, heart, torpedo and cotyledon stages of microspore-derived embryo development (Figure 6A). *BNM3* mRNAs are not detected in non-embryogenic microspore cultures, in freshly isolated microspores and pollen, or in microspores and pollen continuing gametophytic development in culture (Figure 6A). RNA gel blot analysis of developing seeds shows that *BNM3* expression is first detected 14 days after pollination (14 DAP), corresponding to the heart stage of embryo development. *BNM3* expression increases during the early (21 DAP) and mid-cotyledon (28 DAP) stages of embryo development and remains constant thereafter (Figure 6B). *BNM3* transcripts were not detected in any of the non-seed tissues tested, reflecting the low level or absence of transcripts in these tissues.

Example 4: Expression of BNM3 in Vegetative Tissues Promotes Asexual Embryo Formation

In order to determine the function the *Brassica napus* BNM3 proteins, the *BNM3* cDNAs were placed under the control of two separate constitutive promoter constructs, a modified sunflower *POLYUBIQUITIN* promoter construct (hereafter referred to as *UBI:BNM3*) and a double enhanced *35S* promoter construct containing an AMV translational enhancer (hereafter referred to as 35S:*BNM3*), and introduced into arabidopsis. Analysis of the phenotype of the transformants indicates that ectopic over expression of the *BNM3* cDNAs promotes the formation of somatic embryos on vegetative structures such as cotyledons, petioles, leaf blades and the shoot apical meristem (Figure 7). The frequency of transformants producing ectopic embryos, as well as the penetrance of the ectopic embryo phenotype, was greater when the *BNM3* gene was expressed under control of the stronger double enhanced 35S promoter-AMV translational enhancer, as compared to the *POLYUBIQUITIN* promoter. Thus a high threshold level of protein product is required to increase the frequency and penetrance of the ectopic embryo phenotype.

BNM3-derived ectopic embryos contain all of the organ systems and tissue layers found in the developing zygotic embryo. *BNM3*-derived ectopic embryos are bipolar (Figures 7D and E) and consist of an axis, comprised of the hypocotyl and radicle regions, shoot and root meristems, and cotyledons (Figure 7E). In addition, each organ system contains the characteristic radial arrangement of three specialized tissue layers (epidermis, ground parenchyma and provascular tissue) found in zygotic embryos (Figure 7E). Continued expression of the *BNM3* gene within the developing ectopic embryo leads to a reiteration of the embryo-forming process, with the result that new embryos are continuously formed on the surface of pre-existing embryos (Figure 7D and E). These results provide conclusive evidence that expression of a single gene, *BNM3*, is sufficient to initiate a signal transduction cascade leading to the formation of fully differentiated asexually-derived embryos.

Example 5: Expression of BNM3 Increases the Regeneration Capacity of Plant Tissues

We examined the effect of *BNM3* gene expression on the ability of arabidopsis plants to regenerate shoots *in vitro* in the presence or absence of added growth regulators. Leaf, root and hypocotyl explants from 10 day old seedlings of wild-type arabidopsis and transgenic arabidopsis lines expressing *BNM3* under control of the *POLYUBIQUITIN* promoter were placed on media containing growth regulators to induce first callus formation and then shoot organogenesis. Root explants from transgenic lines show at least a 5-fold increase in shoot regeneration in the presence of hormones as compared to wild-type root explants. (Figure 8A). These shoots also developed faster in the transgenic explants as compared to the wild-type. Wild-type leaf and hypocotyl explants responded by producing callus on the cut end of the petiole (Figure 8B). In contrast, explants from transgenic lines immediately produced new shoots (Figure 8B) or roots from the cut end of the petiole. Transgenic explants that initially produced roots eventually also produced shoots.

Transgenic explants were also able to regenerate in absence of added growth regulators. Wild-type leaf and hypocotyl explants placed on medium lacking growth regulators occasionally produced callus or roots at the cut end of the leaf petiole, however shoots did not regenerate from these structures (Figure 8C,D). Wild-type roots greened and formed thickened nodule-like structures at the junction with lateral roots, but did not develop further. In contrast, transgenic explants placed on media lacking growth regulators regenerated shoots either from the cut end of the leaf and hypocotyl explants or from the nodule-like structures of root explants (Figure 8C,D).

The present invention has been described with regard to prefered embodiments. However, it will be obvious to persons skilled in the art that a number of variations and modifications can be made without departing from the scope of the invention as described herein.

References

Anderson, N.L. and Anderson, N.G. (1998). Proteome and proteomics: new technologies, new concepts and new words. Electrophoresis 19, 1853-1861.

Angenent, G.C., Franken, J., Busscher, M., van Dijken, A., van Went, J.L., Dons, H.J.M. and van Tunen, A.J. (1995). A novel class of MADS box genes involved in ovule development in Petunia. Plant Cell 7, 1569-1582.

Aoyama, T. and Chua, N.H. (1997). A glucocorticoid-mediated transcriptional induction system in transgenic plants. Plant J. 2, 397-404.

Baszczynski, C.L., and Fallis, L. (1990). Isolation and nucleotide sequence of a genomic clone encoding a new *Brassica napus* napin gene. Plant Mol. Biol. 14, 633-635.

Binet, M.N., Lepetit, M., Weil, J.H. and Tessier, L.H. (1991). Analysis of a sunflower polyubiquitin promoter by transient expression. Plant Sci. 79, 87-94.

Boutilier, K.A., Gines, M.J., Demoor, J.M., Huang, B., Baszczynski, C.L., Iyer, V.N. and Miki, B.L. (1994). Expression of the BnmNAP subfamily of napin genes coincides with the induction of Brassica microspore embryogenesis. Plant Mol. Biol. 26, 1711-1723.

Brandstatter, I. and Kieber, J.J. (1998). Two genes with similarity to bacterial response regulators are rapidly and specifically induced by cytokinin in Arabidopsis. Plant Cell 10, 1009-1019.

Caddick, M.X., Greenland, A.J., Jepson, I., Krause, K.P., Qu, N., Riddell, K.V., Salter, M.G., Schuch, W., Sonnewald, U., and Tomsett, A.B. (1998). An ethanol inducible gene switch for plants used to manipulate carbon metabolism. Nature Biotech. 16, 177-180.

Chaudury, A.M., Letham, D.S., Craig, S. and Dennis, E.S. (1993). amp-1-a mutant with higher cytokinin levels and altered embryonic pattern, faster vegetative growth, constitutive photomorphogenesis and precocious flowering. Plant J. 4, 907-916.

Clough, S.J. and Bent, A.F. (1998). Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J. 16, 735-743.

Custers, J.B.M, Oldenhof, M.T., Schrauwen, J.A.M., Cordewener, J.H.G., Wullems, G.J. and van Lookeren Campagne, M.M. (1997) Analysis of microspore-specific promoters in tobacco. Plant Mol. Biol 35, 689-699.

Crouch, M.L., Tenbarge, K.M., Simon, A.E. and Ferl, R. (1983). cDNA clones for Brassica napus seed storage proteins: evidence from nucleotide sequence analysis that both subunits of napin are cleaved from a precursor polypeptide. J. Mol. Appl. Genet. 2, 273-283.

Daley, M., Knauf, V.C., Summerfelt, K.R. and Turner, J.C. (1998). Co-transformation with one Agrobacterium tumefaciens strain containing two binary plasmids as a method for producing marker free transgenic plants. Plant Cell Rep. 17, 489-496.

Daniell, T.J., Fordham-Skelton, A.P., Vergani, P. and Edwards, R. (1996). Isolation of a maize cDNA (acession no. Z47554) (PGR 96-013) encoding APETALA-2-like binding domains by complementation cloning of an L-isoaspartyl methyltransferase-deficient mutant of Escherichia coli. Plant Phys. 110, 1435.

Datla, R.S.S., Bekkaoui, F., Hammerlindl, J.K., Pilate, G., Dunstan, D.I. and Crosby, W.L. (1993). Improved high-level constitutive foreign gene expression in plants using an AMV RNA4 untranslated leader sequence. Plant Sci. 94, 139-149.

DeBlock, M. DeBrower, D. and Tenning, P. (1989). Transformation of Brassica napus and Brasica oleracea using Agrobacterium tumefaciens and the expression of the bar and neo genes in the transgenic plants. Plant Physiol. 91: 694-701.

Dellaert, L.M.W. (1981) Comparison of X-ray and fast neutron-induced mutant spectra. Experiments in Arabidopsis thaliana (L.) Heynh. Arabidopsis Inf. Ser., 18, 16-36.

Doan, D. N. P., Linnestad, C., and Olsen, O.-A. (1996). Isolation of molecular markers from the barley endosperm coenocyte and the surrounding nucellus cell layers. Plant Mol. Biol. 31: 877-886.

Elliot, R.C., Betzner, A.S., Huttner, E., Oakes, M.P., Tucker, W.Q.J., Gerentes, D., Perez, P. and Smyth, D.R. (1996). AINTEGUMENTA, an APETALA2-like gene of arabidopsis with pleiotropic roles in ovule development and floral organ growth. Plant Cell 8, 155-168.

Federoff, N., Furtek, D., and Nelson O. (1984). Cloning of the bronze locus in maize by a simple and general procedure using the transposable controlling element Ac. Proc. Natl. Acad. Sci. USA 81, 3825-3829.

Feldman, K.A., Marks, M.D., Christianson, M.L., and Quatrano, R.S. (1989). A dwarf mutant *Arabidopsis* generated by T-DNA insertion mutagenesis. Science 243, 1351-1354.

Fobert, P.R., Miki, B.L., and Iyer, V.N. (1991). Detection of gene regulatory signals in plants revealed by T-DNA-mediated fusions. Plant Mol. Biol. 17, 837-851.

Gatz, C. (1997). Chemical control of gene expression. Ann. Rev. Plant Physiol. Plant Mol. Biol. 48, 89-108.

Gatz, C. and Lenk, I.R.P. (1998). Promoters that respond to chemical inducers. Trends Plant Sci. 3, 352-358.

Guerche, P., Tire, C., Grossi De Sa, F., De Clercq, A., Van Montagu, M. and Krebbers, E. (1990). Differential expression of the Arabidopsis 2S albumin genes and the effect of increasing gene family size. Plant Cell 2, 469-478.

Gustavsson, H.O., Ellerstrom, M., Stulberg, K., Ezcurra, I., Koman, A., Hoglund, A., Rask, L. and Josefsson, L.-G. (1991). Distinct sequence elements in a napin promoter interact in vitro with DNA-binding proteins from Brassica napus. Physiol. Plant 82, 205-212.

Haseloff, J. and Amos, B. (1995). GFP in plants. Trends Genet. 11, 328-329.

Jefferson, R.A. and Bicknell, R. (1996). The potential impacts of apomixis:a molecular genetics approach. In The Impact of Plant Molecular Genetics, B.W.S. Sobral, ed (Boston: Birkhanser), pp. 87-101.

Jefferson, R.A., Kavanagh, T.A. and Bevan, M.W. (1987). GUS fusions: β-glucuronidase as a sensitive and versalite gene fusion marker in higher plants. EMBO J. 6, 3901-3907.

Jofuku, K.D., den Boer, B.G.W., van Montagu, M. and Okamuro, J.K. (1994). Control of Arabidopsis flower and seed development by the homeotic gene APETALA2. Plant Cell 6, 1211-1225.

Kakimoto, T. (1996). CKI7, a histidine kinase homolog implicated in cytokinin signal transduction. Science 274, 982-985.

Keller, W.A., Fan, Z., Pechan, P.,Long, N.,Grainger, J. (1987). An efficient method for culture of isolated microspores of Brassica napus. Proceedings of the 7th International Rapeseed Congress. Poznan, Poland. Vol. 1, 152-157.

Klein, T.M., Wolf, E.D., Wu, R. and Sanford, J.C. (1987). High-velocity microprojectiles for delivering nucleic acids into living cells. Nature 327,70-73

Klimyuk, V.I. and Jones, J.D.G. (1997). AtDMC1, the Arabidopsis homologue of the yeast DMC1 gene: characterization, transposon-induced allelic variation and meiosis-associated expression. Plant J. 11, 1-14.

Klucher, K.M., Chow, H., Reiser, L. and Fischer, R.L. (1996). The AINTEGUMENTA gene of Arabidopsis required for ovule and female gametophyte development is related to the floral homeotic gene APETALA2. Plant Cell 8, 137-153.

Koltunow, A.M., Bicknell, R.A. and Chaudhury, A.M. (1995). Apomixis: molecular strategies for the generation of genetically identical seeds without fertilization. Plant Physiol. 108,1345-1352.

Koltunow, A.M. (1993). Apomixis: embryo sacs and embryos formed without meiosis or fertilization in ovules. Plant Cell 5, 1425-1437.

Korneef, M., Hanhart, C.J. and Thiel, F. (1989). A genetic and phenotypic description of eceriferum (cer) mutants in Arabidopsis thaliana. J. Hered. 80, 118-122.

Lightner J., and Caspar, T. (1988) Seed Mutagenesis of Arabidopsis. In Arabidopsis Protocols, J.M. Martinez-Zapater and J. Salinas eds (Totowa, USA: Humana Press).

Lipshultz, R.J., Fodor, S.P.A., Gingeras, T.R. and Lockhart, D.J. (1999). High density synthetic oligonucleotide arrays. Nature Genetics 21, 20-24.

Lotan, T., Ohto, M., Matsudaira Yee, K., West, M.A.L., Lo, R., Kwong, R.W., Yamagishi, K., Fischer, R.L., Goldberg, R.B. and Harada, J.J. (1998). Arabidopsis LEAFY COTYLEDON1 is sufficient to induce embryo development in vegetative cells. Cell 93, 1195-1205.

Meijer, A.H., Ouwerkerk, P.B.F. and Hoge, J.H.C. (1998). Vectors for transcription factor cloning and target site identification by means of genetic selection in yeast. Yeast 14, 1407-1415.

Moose, S.P. and Sisco, P.H. (1996). Glossy15, an APETALA2-like gene from maize that regulates leaf epidermal cell identity. Genes Dev. 10, 3018-3027.

Mordhorst, A.P., Toonen, M.A.J. and de Vries, S.C. (1997). Plant embryogenesis. Crit. Rev. Plant Sci. 16, 535-576.

Mordhorst, A.P., Voerman, K.J., Hartog, M.V., Meijer, E.A., van Went, J., Koomneef, M., and de Vries, S.C. (1998). Somatic embryogenesis in Arabidopsis thaliana is facilitated by mutations in genes repressing meristematic cell divisions. Genetics 149, 549-563.

Ogas, J., Cheng, J.-C., Sung, R.Z. and Somerville, C. (1997). Cellular differentiation regulated by gibberellin in the Arabidopsis thaliana pickle mutant. Science 277, 91-94.

Ohme-Takagi, M. and Shinshi, H. (1995). Ethylene-inducible DNA binding proteins that interact with an ethylene responsive element. Plant Cell 7, 173-182.

Oldenhof, M.T., de Groot, P.F.M., Visser, J.H., Schrauwen, J.A.M. and Wullems, G.J. (1996). Isolation and characterization of a microspore-specific gene from tobacco. Plant Mol. Biol. 31, 213-225.

Ouellet, T., Rutledge, R.G. and Miki, B.L. (1992). Members of the acetohydroxyacid synthase multigene family of Brassica napus have divergent patterns of expression. Plant J. 2, 321-330.

Ow, D.W., Jacobs, J.D. and Howell, S.H. (1987). Functional regions of the cauliflower mosaic virus 35S RNA promoter determined by use of the firefly luciferase gene as a reporter for promoter activity. Proc. Natl. Acad. Sci. USA 84, 4870-4874.

Ozias-Akins, P., Lubbers, E.L., Hanna, W.W. and McNay, J.W. (1993). Transmission of the apomictic mode of reproduction in Pennisetum: co-inheritance of the trait and molecular markers. Theor. Appl. Genet. 85, 632-638.

Pang, P., Pruitt, R. and Meyerowitz, E. (1988). Molecular cloning, genomic organisation, expression and evolution of the 12S storage protein genes of Arabidopsis thaliana. Plant Mol. Biol. 11, 805-820.

Peacock, W.J., Ming, L., Craig, S., Dennis, E., Chaudury, A.M. (1995). A mutagenesis programme for apomixis genes in Arabidopsis. In Proceedings Symposium on Induced Mutations and Molecular Techniques for Crop Improvement, (Vienna: International Atomic Energy Agency), pp, 117-125

Pechan, P.M., Bartels, D., Brown, D.C.W. and Schell, J. (1991). Messenger-RNA and protein changes associated with induction of Brassica microspore embryogenesis. Planta 184, 161-165.

Roberts, M.R., Foster, G.D., Blundell, R.P., Robinson, S.W., Kumar, A., Draper, J. and Scott, R. (1993). Gametophytic and sporophytic expression of an anther-specific Arabidopsis thaliana gene. Plant J. 3, 111-120.

Rounsley, S.D., Ditta, G.S. and Yanofsky, M.F. (1995). Diverse roles for MADS box genes in Arabidopsis development. Plant Cell 7, 1259-1269.

Salter, M.G., Paine, J.A., Riddell, K.V., Jepson, I., Greenland, A.J., Caddick, M.X., Tomsett, A.B. (1998). Characterisation of the ethanol-inducible alc gene expression system for transgenic plants. Plant Journal 16, 127-132.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, second edition. (Cold Spring Harbor: Cold Spring Harbor Laboratory Press).

Schmidt, E.D.L., Guzzo, F., Toonen, M.A.J. and de Vries, S.C. (1997). A leucine-rich repeat containing receptor-like kinase marks somatic plant cells competent to form embryos. Development. 124, 2049-2062.

Stockinger, E.J., Gilmour, S.J. and Thomashow, M.F. (1997). Arabidopsis thaliana CBF1encodes an AP2 domain-containing transcriptional activator that binds to the C-repeat/DRE, a cis-acting DNA regulatory element that stimulates transcription in response to low temperature and water deficit. Proc. Natl. Acad. Sci. USA 94, 1035-1040.

Taylor, R.L. (1967). The foliar embryos of Malaxis paludosa. Can. J. Bot. 45, 1553-1556.

Treacy, B.K., Hattori, J., Prud'homme, I., Barbour, E., Boutilier, K., Baszczynski, C.L., Huang, B., Johnson, D.A. and Miki, B.L. (1997). Bnm1, a Brassica pollen-specific gene. Plant Mol. Biol. 34, 603-611.

Twell, D., Wing, R., Yamaguchi, J. and McCormick, S. (1989). Isolation and expression of an anther-specific gene from tomato. Mol. Gen. Genet. 217, 240-245.

Ulmasov, T., Murfett, J., Hagen, G. and Guilfoyle, T. (1997). Aux/IAA proteins repress expression of reporter genes containing natural and highly active synthetic auxin response elements. Plant Cell 9, 1963-1971.

van Engelen, F.A., Molthoff, J.W., Conner, A.J., Nap, J.P., Pereira, A. and Stiekema, W.J. (1995). pBINPLUS: an improved plant transformation vector based on pBIN19. Transgenic Res. 4, 288-290.

Vergunst, A.C., de Waal, E.C. and Hooykaas, P.J.J. (1998). Root transformation by Agrobacterium tumefaciens. In Arabidopsis Protocols, J.M. Martinez-Zapater and J. Salinas, eds (Totowa, USA: Humana Press).

Xu, D., McElroy, D., Thornburg, R.W. and Wu, R.C.S. (1993). Systemic induction of a potato pin2 promoter by wounding, methyl jasmonate, and abscisic acid in transgenic rice plants. Plant Mol. Biol. 22, 573-588.

Xu, H., Knox, R.B., Taylor, P.E. and Singh, M.B. (1995). Bcp1, a gene required for male fertility in Arabidopsis. Proc. Natl. Acad. Sci. USA 92, 2106-2110.

Yarbrough, J.A. (1932). Anatomical and developmental studies of the foliar embryos of Bryophyllum calicyinum. Amer. J. Bot. 19, 443-453.

Yeung, E.C. (1995). Structural and developmental patterns in somatic embryogenesis. In In Vitro Embryogenesis in Plants, T.A. Thorpe, ed (Dordrecht: Kluwer Academic Publishers), pp. 205-247.

Zarsky, V., Garrido, D., Eller, N., Tupy, J., Vicente, O., Sch÷ffl, F. and Heberle-Bors, E. (1995). The expression of a small heat shock gene is activated during induction of tobacco pollen embryogenesis by starvation. Plant Cell Environ. 18, 139-147.

Zhou, J., Tang, X. and Martin, G.B. (1997). The Pto kinase conferring resistance to tomato bacterial speck disease interacts with proteins that bind a cis-element of pathogenesis-related genes. EMBO J. 16, 3207-3218.

SEQUENCE LISTING

<110> CPRO-DLO
Agriculture and Agri-food Canada

<120> Use of the BNM3 Transcriptional Activator to Control Plant Embryogenesis and Regeneration Processes

<130> E158138

<140>
<141>

<160> 12

<170> PatentIn Ver. 2.1

<210> 1
<211> 2014
<212> DNA
<213> Brassica napus

<400> 1
gttcatctct cttctttaag accaaaacct ttttctcctc ctcttcatgc atgaacccta 60
actaagttct tcttctttta ccttttacca agaactcgtt agatcactct ctgaactcaa 120
tgaataataa ctggttaggc ttttctctct ctccttatga acaaaatcac catcgtaagg 180
acgtctactc ttccaccacc acaaccgtcg tagatgtcgc cggagagtac tgttacgatc 240
cgaccgctgc ctccgatgag tcttcagcca tccaaacatc gtttccttct ccctttggtg 300
tcgtcgtcga tgctttcacc agagacaaca atagtcactc ccgagattgg gacatcaatg 360
gttgtgcatg caataacatc cacaacgatg agcaagatgg accaaagctt gagaatttcc 420
ttggccgcac caccacgatt tacaacacca acgaaaacgt tggagatgga agtggaagtg 480
gctgttatgg aggaggagac ggtggtggtg gctcactagg actttcgatg ataaagacat 540
ggctgagaaa tcaacccgtg gataatgttg ataatcaaga aaatggcaat gctgcaaaag 600
gcctgtccct ctcaatgaac tcatctactt cttgtgataa caacaacgac agcaataaca 660
acgttgttgc ccaagggaag actattgatg atagcgttga agctacaccg aagaaaacta 720
ttgagagttt tggacagagg acgtctatat accgcggtgt tacaaggcat cggtggacag 780
gaagatatga ggcacattta tgggataata gttgtaaaag agaaggccaa acgcgcaaag 840
gaagacaagt ttatttggga ggttatgaca aagaagaaaa agcagctagg gcttatgatt 900
tagccgcact caagtattgg ggaaccacca ctactactaa cttccccatg agcgaatatg 960
aaaaagaggt agaagagatg aagcacatga caaggcaaga gtatgttgcc tcactgcgca 1020
ggaaaagtag tggtttctct cgtggtgcat cgatttatcg tggagtaaca agacatcacc 1080
aacatggaag atggcaagct aggataggaa gagtcgccgg taacaaagac ctctacttgg 1140
gaacttttgg cacacaagaa gaagctgcag aggcatacga cattgcggcc atcaaattca 1200
gaggattaac cgcagtgact aacttcgaca tgaacagata caacgttaaa gcaatcctcg 1260
aaagccctag tcttcctatt ggtagcgccg caaaacgtct caaggaggct aaccgtccgg 1320
ttccaagtat gatgatgatc agtaataacg tttcagagag tgagaatagt gctagcggtt 1380
ggcaaaacgc tgcggttcag catcatcagg gagtagattt gagcttattg caccaacatc 1440
aagagaggta caatggttat tattacaatg gaggaaactt gtcttcggag agtgctaggg 1500

cttgtttcaa acaagaggat gatcaacacc atttcttgag caacacgcag agcctcatga 1560
ctaatatcga tcatcaaagt tctgtttcgg atgattcggt tactgtttgt ggaaatgttg 1620
ttggttatgg tggttatcaa ggatttgcag ccccggttaa ctgcgatgcc tacgctgcta 1680
gtgagtttga ttataacgca agaaaccatt attactttgc tcagcagcag cagacccagc 1740
agtcgccagg tggagatttt cccgcggcaa tgacgaataa tgttggctct aatatgtatt 1800
accatgggga aggtggtgga gaagttgctc caacatttac agtttggaac gacaattaga 1860
aaaaatagtt aaagatcttt agttatatgc gttgttgtgt gctggtgaac agtgtgatac 1920
tttgattatg ttttttctt tctcttttc tttttcttgg ttaatttctt aagacttatt 1980
tttagtttcc attagttgga taaattttca gact 2014

<210> 2
<211> 579
<212> PRT
<213> Brassica napus

<400> 2
Met Asn Asn Asn Trp Leu Gly Phe Ser Leu Ser Pro Tyr Glu Gln Asn
1 5 10 15

His His Arg Lys Asp Val Tyr Ser Ser Thr Thr Thr Thr Val Val Asp
20 25 30

Val Ala Gly Glu Tyr Cys Tyr Asp Pro Thr Ala Ala Ser Asp Glu Ser
35 40 45

Ser Ala Ile Gln Thr Ser Phe Pro Ser Pro Phe Gly Val Val Val Asp
50 55 60

Ala Phe Thr Arg Asp Asn Asn Ser His Ser Arg Asp Trp Asp Ile Asn
65 70 75 80

Gly Cys Ala Cys Asn Asn Ile His Asn Asp Glu Gln Asp Gly Pro Lys
85 90 95

Leu Glu Asn Phe Leu Gly Arg Thr Thr Thr Ile Tyr Asn Thr Asn Glu
100 105 110

Asn Val Gly Asp Gly Ser Gly Ser Gly Cys Tyr Gly Gly Gly Asp Gly
115 120 125

Gly Gly Gly Ser Leu Gly Leu Ser Met Ile Lys Thr Trp Leu Arg Asn
130 135 140

Gln Pro Val Asp Asn Val Asp Asn Gln Glu Asn Gly Asn Ala Ala Lys
145 150 155 160

Gly Leu Ser Leu Ser Met Asn Ser Ser Thr Ser Cys Asp Asn Asn Asn

```
                165                 170                 175

Asp Ser Asn Asn Asn Val Val Ala Gln Gly Lys Thr Ile Asp Asp Ser
            180                 185                 190

Val Glu Ala Thr Pro Lys Lys Thr Ile Glu Ser Phe Gly Gln Arg Thr
        195                 200                 205

Ser Ile Tyr Arg Gly Val Thr Arg His Arg Trp Thr Gly Arg Tyr Glu
    210                 215                 220

Ala His Leu Trp Asp Asn Ser Cys Lys Arg Glu Gly Gln Thr Arg Lys
225                 230                 235                 240

Gly Arg Gln Val Tyr Leu Gly Gly Tyr Asp Lys Glu Glu Lys Ala Ala
                245                 250                 255

Arg Ala Tyr Asp Leu Ala Ala Leu Lys Tyr Trp Gly Thr Thr Thr Thr
            260                 265                 270

Thr Asn Phe Pro Met Ser Glu Tyr Glu Lys Glu Val Glu Glu Met Lys
        275                 280                 285

His Met Thr Arg Gln Glu Tyr Val Ala Ser Leu Arg Arg Lys Ser Ser
    290                 295                 300

Gly Phe Ser Arg Gly Ala Ser Ile Tyr Arg Gly Val Thr Arg His His
305                 310                 315                 320

Gln His Gly Arg Trp Gln Ala Arg Ile Gly Arg Val Ala Gly Asn Lys
                325                 330                 335

Asp Leu Tyr Leu Gly Thr Phe Gly Thr Gln Glu Glu Ala Ala Glu Ala
            340                 345                 350

Tyr Asp Ile Ala Ala Ile Lys Phe Arg Gly Leu Thr Ala Val Thr Asn
        355                 360                 365

Phe Asp Met Asn Arg Tyr Asn Val Lys Ala Ile Leu Glu Ser Pro Ser
    370                 375                 380

Leu Pro Ile Gly Ser Ala Ala Lys Arg Leu Lys Glu Ala Asn Arg Pro
385                 390                 395                 400

Val Pro Ser Met Met Met Ile Ser Asn Asn Val Ser Glu Ser Glu Asn
                405                 410                 415

Ser Ala Ser Gly Trp Gln Asn Ala Ala Val Gln His His Gln Gly Val
```

```
            420                 425                 430

Asp Leu Ser Leu Leu His Gln His Gln Glu Arg Tyr Asn Gly Tyr Tyr
        435                 440                 445

Tyr Asn Gly Gly Asn Leu Ser Ser Glu Ser Ala Arg Ala Cys Phe Lys
    450                 455                 460

Gln Glu Asp Asp Gln His His Phe Leu Ser Asn Thr Gln Ser Leu Met
465                 470                 475                 480

Thr Asn Ile Asp His Gln Ser Ser Val Ser Asp Asp Ser Val Thr Val
                485                 490                 495

Cys Gly Asn Val Val Gly Tyr Gly Gly Tyr Gln Gly Phe Ala Ala Pro
            500                 505                 510

Val Asn Cys Asp Ala Tyr Ala Ala Ser Glu Phe Asp Tyr Asn Ala Arg
        515                 520                 525

Asn His Tyr Tyr Phe Ala Gln Gln Gln Gln Thr Gln Gln Ser Pro Gly
    530                 535                 540

Gly Asp Phe Pro Ala Ala Met Thr Asn Asn Val Gly Ser Asn Met Tyr
545                 550                 555                 560

Tyr His Gly Glu Gly Gly Gly Glu Val Ala Pro Thr Phe Thr Val Trp
                565                 570                 575

Asn Asp Asn
```

<210> 3
<211> 2011
<212> DNA
<213> Brassica napus

<400> 3
ttcttctttt accttttacc aagaactcgt tagatcattt tctgaactcg atgaataata 60
actggttagg cttttctctc tctccttatg aacaaaatca ccatcgtaag gacgtctgct 120
cttccaccac cacaaccgcc gtagatgtcg ccggagagta ctgttacgat ccgaccgctg 180
cctccgatga gtcttcagcc atccaaacat cgtttccttc tccctttggt gtcgtcctcg 240
atgctttcac cagagacaac aatagtcact cccgagattg ggacatcaat ggtagtgcat 300
gtaataacat ccacaatgat gagcaagatg gaccaaaact tgagaatttc cttggccgca 360
ccaccacgat ttacaacacc aacgaaaacg ttggagatat cgatggaagt gggtgttatg 420
gaggaggaga cggtggtggt ggctcactag gactttcgat gataaagaca tggctgagaa 480

```
atcaacccgt ggataatgtt gataatcaag aaaatggcaa tggtgcaaaa ggcctgtccc 540
tctcaatgaa ctcatctact tcttgtgata acaacaacta cagcagtaac aaccttgttg 600
cccaagggaa gactattgat gatagcgttg aagctacacc gaagaaaact attgagagtt 660
ttggacagag gacgtctata taccgcggtg ttacaaggca tcggtggaca ggaagatatg 720
aggcacattt atgggataat agttgtaaac gagaaggcca aacgcgcaaa ggaagacaag 780
tttatttggg aggttatgac aaagaagaaa aagcagctag ggcttatgat ttagccgcac 840
tcaagtattg gggaaccacc actactacta acttccccat gagcgaatat gagaaagaga 900
tagaagagat gaagcacatg acaaggcaag agtatgttgc ctcacttcgc aggaaaagta 960
gtggtttctc tcgtggtgca tcgatttatc gtggagtaac aagacatcac caacatggaa 1020
gatggcaagc taggatagga agagtcgccg gtaacaaaga cctctacttg ggaacttttg 1080
gcacacaaga agaagctgca gaggcatacg acattgcggc catcaaattc agaggattaa 1140
ccgcagtgac taacttcgac atgaacagat acaacgttaa agcaatcctc gaaagcccta 1200
gtcttcctat tggtagcgcc gcaaaacgtc tcaaggaggc taaccgtccg gttccaagta 1260
tgatgatgat cagtaataac gtttcagaga gtgagaataa tgctagcggt tggcaaaacg 1320
ctgcggttca gcatcatcag ggagtagatt tgagcttatt gcagcaacat caagagaggt 1380
acaatggtta ttattacaat ggaggaaact tgtcttcgga gagtgctagg gcttgtttca 1440
aacaagagga tgatcaacac catttcttga gcaacacgca gagcctcatg actaatatcg 1500
atcatcaaag ttctgtttca gatgattcgg ttactgtttg tggaaatgtt gttggttatg 1560
gtggttatca aggatttgca gccccggtta actgcgatgc ctacgctgct agtgagtttg 1620
actataacgc aagaaaccat tattactttg ctcagcagca gcagacccag cattcgccag 1680
gaggagattt tcccgcggca atgacgaata atgttggctc taatatgtat taccatgggg 1740
aaggtggtgg agaagttgct ccaacattta cagtttggaa cgacaattag aaataatagt 1800
taaagatctt tagttatatg cgttgttgtg tggtgttgaa cagtttgata ctttgattat 1860
gttttttttt ctcttttttca ttttgttggt tagtttctta agacttattt tttgtttcca 1920
ttagttggat aaattttcgg acttaagggt cacttctgtt ctgacttctg tctaatacag 1980
aaaagttttc ataaaaaaaa aaaaaaaaaa a                                  2011
```

<210> 4
<211> 579
<212> PRT
<213> Brassica napus

<400> 4

```
Met Asn Asn Asn Trp Leu Gly Phe Ser Leu Ser Pro Tyr Glu Gln Asn
 1               5                  10                  15

His His Arg Lys Asp Val Tyr Ser Ser Thr Thr Thr Thr Val Val Asp
            20                  25                  30

Val Ala Gly Glu Tyr Cys Tyr Asp Pro Thr Ala Ala Ser Asp Glu Ser
        35                  40                  45

Ser Ala Ile Gln Thr Ser Phe Pro Ser Pro Phe Gly Val Val Val Asp
    50                  55                  60

Ala Phe Thr Arg Asp Asn Asn Ser His Ser Arg Asp Trp Asp Ile Asn
65                  70                  75                  80
```

Gly Cys Ala Cys Asn Asn Ile His Asn Asp Glu Gln Asp Gly Pro Lys
85 90 95

Leu Glu Asn Phe Leu Gly Arg Thr Thr Thr Ile Tyr Asn Thr Asn Glu
100 105 110

Asn Val Gly Asp Gly Ser Gly Ser Gly Cys Tyr Gly Gly Gly Asp Gly
115 120 125

Gly Gly Gly Ser Leu Gly Leu Ser Met Ile Lys Thr Trp Leu Arg Asn
130 135 140

Gln Pro Val Asp Asn Val Asp Asn Gln Glu Asn Gly Asn Ala Ala Lys
145 150 155 160

Gly Leu Ser Leu Ser Met Asn Ser Ser Thr Ser Cys Asp Asn Asn Asn
165 170 175

Asp Ser Asn Asn Asn Val Val Ala Gln Gly Lys Thr Ile Asp Asp Ser
180 185 190

Val Glu Ala Thr Pro Lys Lys Thr Ile Glu Ser Phe Gly Gln Arg Thr
195 200 205

Ser Ile Tyr Arg Gly Val Thr Arg His Arg Trp Thr Gly Arg Tyr Glu
210 215 220

Ala His Leu Trp Asp Asn Ser Cys Lys Arg Glu Gly Gln Thr Arg Lys
225 230 235 240

Gly Arg Gln Val Tyr Leu Gly Gly Tyr Asp Lys Glu Glu Lys Ala Ala
245 250 255

Arg Ala Tyr Asp Leu Ala Ala Leu Lys Tyr Trp Gly Thr Thr Thr Thr
260 265 270

Thr Asn Phe Pro Met Ser Glu Tyr Glu Lys Glu Val Glu Glu Met Lys
275 280 285

His Met Thr Arg Gln Glu Tyr Val Ala Ser Leu Arg Arg Lys Ser Ser
290 295 300

Gly Phe Ser Arg Gly Ala Ser Ile Tyr Arg Gly Val Thr Arg His His
305 310 315 320

Gln His Gly Arg Trp Gln Ala Arg Ile Gly Arg Val Ala Gly Asn Lys
325 330 335

```
Asp Leu Tyr Leu Gly Thr Phe Gly Thr Gln Glu Glu Ala Ala Glu Ala
            340                 345                 350

Tyr Asp Ile Ala Ala Ile Lys Phe Arg Gly Leu Thr Ala Val Thr Asn
        355                 360                 365

Phe Asp Met Asn Arg Tyr Asn Val Lys Ala Ile Leu Glu Ser Pro Ser
    370                 375                 380

Leu Pro Ile Gly Ser Ala Ala Lys Arg Leu Lys Glu Ala Asn Arg Pro
385                 390                 395                 400

Val Pro Ser Met Met Met Ile Ser Asn Asn Val Ser Glu Ser Glu Asn
                405                 410                 415

Ser Ala Ser Gly Trp Gln Asn Ala Ala Val Gln His His Gln Gly Val
            420                 425                 430

Asp Leu Ser Leu Leu His Gln His Gln Glu Arg Tyr Asn Gly Tyr Tyr
        435                 440                 445

Tyr Asn Gly Gly Asn Leu Ser Ser Glu Ser Ala Arg Ala Cys Phe Lys
    450                 455                 460

Gln Glu Asp Asp Gln His His Phe Leu Ser Asn Thr Gln Ser Leu Met
465                 470                 475                 480

Thr Asn Ile Asp His Gln Ser Ser Val Ser Asp Asp Ser Val Thr Val
                485                 490                 495

Cys Gly Asn Val Val Gly Tyr Gly Gly Tyr Gln Gly Phe Ala Ala Pro
            500                 505                 510

Val Asn Cys Asp Ala Tyr Ala Ala Ser Glu Phe Asp Tyr Asn Ala Arg
        515                 520                 525

Asn His Tyr Tyr Phe Ala Gln Gln Gln Gln Thr Gln Gln Ser Pro Gly
    530                 535                 540

Gly Asp Phe Pro Ala Ala Met Thr Asn Asn Val Gly Ser Asn Met Tyr
545                 550                 555                 560

Tyr His Gly Glu Gly Gly Gly Glu Val Ala Pro Thr Phe Thr Val Trp
                565                 570                 575

Asn Asp Asn
```

<210> 5
<211> 4873
<212> DNA
<213> Brassica napus

<220>
<221> intron
<222> (1846)..(2298)

<220>
<221> intron
<222> (2720)..(2952)

<220>
<221> intron
<222> (3036)..(3160)

<220>
<221> intron
<222> (3170)..(3314)

<220>
<221> intron
<222> (3404)..(3553)

<220>
<221> intron
<222> (3628)..(3797)

<220>
<221> intron
<222> (3849)..(3961)

<220>
<221> intron
<222> (4039)..(4148)

<220>
<221> misc_feature
<222> (1620)..(1622)
<223> start codon

<220>
<221> misc_feature
<222> (4856)..(4858)

<223> stop codon

<400> 5

atctctccac cgattcgtta cccagtgctt gaaaatatga tgactacgaa tcaattaaat 60
ggagaagctc cactgcttgt gtaggtggaa gctcaagcaa caaccggaaa cctcggcgtt 120
atcgggagtt agcatcgtta tttgccaaaa tttccgccgc agagatgaaa cgattcaaga 180
gaaaccctca aataggttag ccataaaaca gtgaattagt atgatttaag agataagaag 240
agaagatgag ttcaagaaaa gaaatactca catctattta tactgtttac acaccgcctt 300
tcagatctaa gcaaagcatt gaagatgaat cgtggaggag agttaatagg atttaacaca 360
aagccattaa ccaaaccgtt gcaggtcggg agacgaaccg caaaagtcac gcctagccgt 420
cgcacgaaga ggagcgatga atttcgtttt ctcgctgcag tcgtattagg gatagacgga 480
gctcattatc gttgggccgg aaacacttct aatctcacag cccatgaaca cactaaagaa 540
cgaaaccgaa aatgtttgaa gtttaatgaa acgtgcggtt tgccttatgg acacatgtca 600
ttacgatatg aaatgattta tctacgtgga tcataggtgt ctctctaagg agagagcaaa 660
cctatacttt atataaatag atttgtatca ttctaagagg tgtttaagat ttttgcataa 720
atattaaaaa aaaatacaaa tttttatgta attagttttg gttacataaa ataacattaa 780
ataaaattaa ttcaaccaat aaaaaaatac ggtattttat aattggtcaa aaataaaaat 840
aaaacattaa atttcaccta gaattacgag aatgtcactt attttgaaac aaaatcaaaa 900
tctttaaaca tcaattaaac tgatacggat ggagtatata tctttacaga gaacatatat 960
atatgttttt cttgtaagcg tccatctctt cttagtcatg tagttcaaat accagctgca 1020
gtaaaaccat gaatatttga atttgttgta aaatattcga agcgactact gcacgtttgg 1080
aagcaaaacg ccaaacgcaa tcgctcgctc ggtcataggg tcacacatac acatgtgact 1140
agcattatgg gtcttaattc aacagcgagt gattttggga tttattatta gttctcgtgt 1200
tactctcact ttaacacaaa gtcactaacc ttatttacac atgaagagag gtttgaaagg 1260
gcttttgact gattaattat aatgtattaa accaaactag aattaagaga ttaggcattg 1320
aattacatta ccaccaccac ccaccattca aaccgaccaa tacatctcca cagttttcaa 1380
gtaaaacaac ttttttttgt tgttccttcg gaatttaaat aaatattcgt ttatataaat 1440
gcgcatgata tgacgcctcg gaagaaatga aacattatat ctttgacttt tcttctccta 1500
gttcatctct cttctttaag accaaaacct ttttctcctc ctcttcatgc atgaacccta 1560
actaagttct tcttctttta ccttttacca agaactcgtt agatcactct ctgaactcaa 1620
tgaataataa ctggttaggc ttttctctct ctccttatga acaaaatcac catcgtaagg 1680
acgtctactc ttccaccacc acaaccgtcg tagatgtcgc cggagagtac tgttacgatc 1740
cgaccgctgc ctccgatgag tcttcagcca tccaaacatc gtttccttct ccctttggtg 1800
tcgtcgtcga cgctttcacc agagacaaca atagtcactc ccgaggttat tgttttagaa 1860
ctacttgttt ttttttgatt tgtttatttg tttagtttcc tcttcttcca atgcgtagaa 1920
caaagaccaa tacacacgca cgcatactag ccctattttt tccttgggct tatttatcga 1980
tttcatttat tttgagaata tcaatgtgtg gggtttgatg tttgtttgca tatagtaata 2040
ctaaaacata tgccagttat acatagattt tttttaaaga tatacatgga tatgaaatga 2100
aatttgacat ttcctccttt attcaatatc ataatatgat cacatacatg tgtacctttt 2160
gatttgtata tttgtttctt acagttgaag gagagaatag ccaaataccc atttgtatat 2220
tatagatcgg tgatgaaaag taaatttaac aaattatgat aatataggcc attaatcttt 2280
gatttttttt ctttatagat tgggacatca atggttgtgc atgcaataac atccacaacg 2340
atgagcaaga tggaccaaag cttgagaatt tccttggccg caccaccacg atttacaaca 2400
ccaacgaaaa cgttggagat ggaagtggaa gtggctgtta tggaggagga gacggtggtg 2460
gtggctcact aggactttcg atgataaaga catggctgag aaatcaaccc gtggataatg 2520
ttgataatca agaaaatggc aatgctgcaa aaggcctgtc cctctcaatg aactcatcta 2580
cttcttgtga taacaacaac gacagcaata acaacgttgt tgcccaaggg aagactattg 2640
atgatagcgt tgaagctaca ccgaagaaaa ctattgagag ttttggacag aggacgtcta 2700

tataccgcgg tgttacaagg tgcccttcat ttatttaatt aaaatgtgta aaatgtcgct 2760
tgaattgtta tcttcttggt aaagtctggg acattgatct aatggctctg ttgcgagagt 2820
gctaccgaat ggtccttgat atatagtatc aaagagagat attgttatta tgggcttata 2880
tagaataata catatatata tatatataca tggtagctgt tgatgacatg tatgttcgta 2940
ttaaatgata aggcatcggt ggacaggaag atatgaggca catttatggg ataatagttg 3000
taaaagagaa ggccaaacgc gcaaaggaag acaaggtata tatatattca ttgataattt 3060
gatcatattt tcatacacga tttactttca aactaatata ggtttttcga tcattgttca 3120
tgtttttatc aaaatttgca cctggtggtt gtcttctcag tttatttggg taagtaattt 3180
attataaatt ggacgaagct gtgatgggta aatctaaatt atataatcaa atttgtttat 3240
tttttgtgta tacattcatt atataatcaa aatagcgata cgatctacat tcaattgttg 3300
tctatatcat gcaggaggtt atgacaaaga agaaaaagca gctagggctt atgatttagc 3360
cgcactcaag tattggggaa ccaccactac tactaacttc cccgtaagtc aatcaatgtt 3420
gtacaagatt tcataactta gaaccaattt tattcttttt ttataagatg ctattatctt 3480
attattaatt gccatgttta tatcgttaca tttattacaa taaaaagtac ttttggtttg 3540
atataatatg tagatgagcg aatatgaaaa agaggtagaa gagatgaagc acatgacaag 3600
gcaagagtat gttgcctcac tgcgcaggta tataatggaa cttctgatat tattgcatat 3660
ggcatctatt attatacatg tatattagta ttttatatat agaacccatc acgctcacgt 3720
ttatatttaa aaatatgtcc gtattcacgt cagattatca gcatacacct atatataata 3780
gacattaaaa tatgcaggaa aagtagtggt ttctctcgtg gtgcatcgat ttatcgtgga 3840
gtaacaaggt attcatacag agagaacgaa tcctattttg ttacgtacat atatattaaa 3900
aaatataatt ataagatatc acattttata ttatgaatat ttcttctaat gggtccaaaa 3960
gacatcacca acatggaaga tggcaagcta ggataggaag agtcgccggt aacaaagacc 4020
tctacttggg aacttttggt acgtttagtc ttctcttact aaacttcaca atcaaatcta 4080
taacaaaaga tatcaactaa aaactacaac atatatctaa gtaagctgta catatattat 4140
atatgaaggc acacaagaag aagctgcaga ggcatacgac attgcggcca tcaaattcag 4200
aggattaacc gcagtgacta acttcgacat gaacagatac aacgttaaag caatcctcga 4260
aagccctagt cttcctattg gtagcgccgc aaaacgtctc aaggaggcta accgtccggt 4320
tccaagtatg atgatgatca gtaataacgt ttcagagagt gagaatagtg ctagcggttg 4380
gcaaaacgct gcggttcagc atcatcaggg agtagatttg agcttattgc accaacatca 4440
agagaggtac aatggttatt attacaatgg aggaaacttg tcttcggaga gtgctagggc 4500
ttgtttcaaa caagaggatg atcaacacca tttcttgagc aacacgcaga gcctcatgac 4560
taatatcgat catcaaagtt ctgtttcgga tgattcggtt actgtttgtg gaaatgttgt 4620
tggttatggt ggttatcaag gatttgcagc cccggttaac tgcgatgcct acgctgctag 4680
tgagtttgat tataacgcaa gaaaccatta ttacttttgct cagcagcagc agacccagca 4740
gtcgccaggt ggagattttc ccgcggcaat gacgaataat gttggctcta atatgtatta 4800
ccatggggaa ggtggtggag aagttgctcc aacatttaca gtttggaacg acaattagaa 4860
aaaatagtta aag 4873

<210> 6
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer

<400> 6

gaggcagcgg tcggatcgta acagtactct 30

<210> 7
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer

<400> 7
cataaggaga gagagaaaag cctaaccagt 30

<210> 8
<211> 19
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer

<400> 8
accaagaact cgttagatc 19

<210> 9
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer

<400> 9
aacgcatata actaaagatc 20

<210> 10
<211> 26
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer

<400> 10

ccatggatcc agagacgaag cgaaac 26

<210> 11
<211> 26
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer

<400> 11
actccatgga taataactgg ttaggc 26

<210> 12
<211> 26
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer

<400> 12
aaattctcaa gctttggtcc atcttg 26

CLAIMS

1. An isolated DNA molecule comprising a nucleotide sequence that hybridizes to SEQ ID NO:5 under stringent conditions.

2. The isolated DNA molecule of claim 1 wherein said isolated DNA molecule comprises at least 23 contiguous nucleotides of SEQ ID NO:5.

3. The isolated DNA molecule of claim 1 wherein said isolated DNA molecule comprises a nucleotide sequence that is at least 70% homologous with the nucleotide sequence defined by SEQ ID NO:5.

4. An isolated DNA molecule comprising a nucleic acid sequence encoding a protein, wherein said protein when present at a sufficient level within a plant cell renders said cell embryogenic, increases the regenerative capacity of said plant cell, or both renders said plant cell embryogenic and increases the regenerative capacity of said plant cell, said isolated DNA molecule having at least 70% homology within a nucleotide sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3 and SEQ ID NO:5.

5. The isolated DNA molecule of claim 4 comprising a nucleotide sequence that ~~hybridizes to nucleotides 1620-4873 of SEQ ID NO:5 under stringent~~ conditions.

6. The isolated DNA molecule of claim 4 comprising a nucleotide sequence that hybridizes to the nucleotide sequence of SEQ ID NO:1 under stringent conditions.

7. The isolated DNA molecule of claim 4 comprising a nucleotide sequence that hybridizes to the nucleotide sequence of SEQ ID NO:3 under stringent conditions.

8. The isolated DNA molecule of claim 6, wherein said DNA encodes a protein as defined by SEQ ID NO:2.

9. The isolated DNA molecule of claim 7, wherein said DNA encodes a protein as defined by SEQ ID NO:4.

10. A vector comprising the isolated DNA molecule as claimed in any one of claims 1 to 9, wherein said isolated DNA molecule is under control of a regulatory element that directs expression of said DNA in a plant cell.

11. The vector of claim 10, wherein said regulatory element is a constitutive regulatory element

12. The vector of claim 10, wherein said regulatory element is an inducible regulatory element.

13. The vector of claim 10, wherein said regulatory element is a tissue specific regulatory element

14. The vector of claim 10, wherein said regulatory element is an developmentally active regulatory element.

15. A transformed plant cell comprising the vector of any one of claims 10 to 14.

16. A transformed plant comprising the vector of any one of claims 10 to 14.

17. A seed obtained from the transformed plant of claim 16.

18 An isolated protein encoded by the isolated DNA molecule as claimed in any one of claims 4 to 9.

19. A method of producing asexually derived embryos comprising:

i) transforming a plant cell with the vector of any one of claims 10 to 14;

ii) growing said plant cell to produce transformed tissue;

iii) selecting said transformed tissue for occurrence of said isolated DNA molecule; and

iv) assaying said transformed plant for asexual embryo production.

20. The method of claim 19 wherein the step of assaying involves assaying for adventitious embryony.

21. The method of claim 19, wherein the step of assaying involves assaying for somatic embryos.

22. The method of claim 19, wherein the step of assaying involves assaying for gametophytic embryos.

23. The method of claim 19, wherein the step of assaying involves assaying for haploid parthenogenesis of the embryo sac.

24. The method of claim 19, wherein the step of assaying involves assaying for diplospory.

25 A method of modifying the regenerative capacity of a plant comprising

i) transforming a plant cell with the vector of any one of claims 10 to 14;

ii) growing said transformed plant cell to produce transformed tissue; and

iii) assaying said transformed plant tissue for enhanced regeneration as compared to wild-type tissue.

26. The method of claim 25, wherein the step of growing said transformed plant cell, the step of assaying said transformed plant tissue, or both the step of growing said transformed plant cell and the step of assaying said transformed plant tissue are carried out in the absence of a growth regulator.

27. A method of selecting a transformed plant comprising;

i) transforming a normally non-regenerative plant with a vector of any one of claims 10 to 14; and

ii) determining whether said transformed plant is able to regenerate under conditions in which said normally non-regenerative plant does not regenerate.

28. The isolated DNA molecule of claim 1 comprising a DNA sequence that hybridizes to nucleotides 1-1619 of SEQ ID NO:5 under stringent conditions.

29. The isolated DNA molecule of claim 1 wherein said isolated molecule comprises at least 22 contiguous nucleotides within nucleotides 1-1619 of SEQ ID NO:5.

30. A vector comprising the isolated DNA molecule of either claim 28 or 29 operably associated with a gene of interest, wherein said isolated DNA molecule directs the expression of said gene of interest within a plant cell.

31. The vector as defined by claim 30, wherein said gene of interest is heterologous with respect to the isolated DNA molecule.

32. The vector as defined by claim 31, wherein said gene of interest is selected from the group consisting of a pharmaceutically active protein, antibody,

industrial enzyme, protein supplement, nutraceutical, storage protein, animal feed and animal feed supplement.

33. A transformed plant cell comprising the vector of either claim 30, 31 or 32.

34. A transformed plant comprising the vector of either claim 30, 31 or 32.

35. A seed obtained from the transformed plant of claim 34.

36. A method for directing the expression of a gene of interest within a developing embryo of a plant comprising transforming said plant with the vector as defined by either claim 30, 31 or 32.

37. A use of a nucleotide sequence as defined in any one of claims 4, 5, 6 or 7 as a selectable marker.

38. A method of producing asexually derived embryos comprising:

i) transiently transforming a plant cell with the vector of any one of claims 10 to 14, or introducing into said plant cell the protein of claim 18, to produce a modified plant cell;

ii) growing said modified plant cell to produce tissue; and

iii) assaying said tissue for asexual embryo formation.

39. The method of claim 38 wherein the step of assaying involves assaying for adventitious embryony.

40. The method of claim 38, wherein the step of assaying involves assaying for somatic embryos.

41. The method of claim 38, wherein the step of assaying involves assaying for gametophytic embryos.

42. The method of claim 38, wherein the step of assaying involves assaying for haploid parthenogenesis of the embryo sac.

43. The method of claim 38, wherein the step of assaying involves assaying for diplospory.

44 A method of modifying the regenerative capacity of a plant comprising

i) transiently transforming a plant cell with the vector of any one of claims 10 to 14 or introducing into said plant cell the protein of claim 18, to produce a modified plant cell;
ii) growing said modified plant cell to produce tissue; and
iii) assaying said tissue for enhanced regeneration as compared to wild-type tissue.

45. The method of claim 44, wherein the step of growing said modified plant cell, the step of assaying said tissue, or both the step of growing said modified plant cell and the step of assaying said tissue are carried out in the absence of a growth regulator.

46. A method of producing an apomictic plant comprising:

i) transforming a plant with the vector of any one of claims 10 to 14, to produce a transformed plant;
ii) selecting said transformed plant for occurrence of said isolated DNA molecule; and
iii) assaying said transformed plant for asexual embryo production.

47. The method of claim 46 wherein the step of assaying involves assaying for adventitious embryony.

48. The method of claim 46, wherein the step of assaying involves assaying for somatic embryos.

49. The method of claim 46, wherein the step of assaying involves assaying for gametophytic embryos.

50. The method of claim 46, wherein the step of assaying involves assaying for parthenogenesis of the embryo sac.

51 A method of modifying the regenerative capacity of a plant comprising

i) transiently transforming a plant cell with the vector of any one of claims 10 to 14, or introducing into said plant cell the protein of claim 18;
ii) growing said plant cell to form tissue; and
iii) assaying said tissue for enhanced regeneration as compared to wild-type tissue.

52. The method of claim 51, wherein the step of growing said plant cell, the step of assaying said tissue, or both the step of growing said plant cell and the step of assaying said tissue are carried out in the absence of a growth regulator.

53. A method of selecting a modified plant comprising;

i) transiently transforming a normally non-regenerative plant with a vector of any one of claims 10 to 14, or introducing into said normally non-regenerative plant the protein of claim 18, to produce said modified plant; and
ii) determining whether said modified plant is able to regenerate under conditions in which said normally non-regenerative plant does not germinate.

54. An isolated DNA molecule comprising a sequence encoding a protein consisting of two AP2 DNA binding domains, which when said protein is expressed at a sufficient level in a plant cell, renders said cell embryogenic, or increase the regenerative capacity of said plant cell, or both renders said cell embryogenic and increase the regenerative capacity of said plant cell.

55. A method of producing a protein of interest comprising

i) transforming a plant with at least one vector, said at least one vector selected from any one of claims 10 to 14 to produce a transformed plant;

ii) selecting said transformed plant for occurrence of said isolated DNA molecule; and

iv) growing said transformed plant in order to produce said protein of interest, wherein expression of said protein of interest is induced by the expression product of said isolated DNA.

56 The method of claim 55, wherein said transformed plant is transformed with a second vector comprising a nucleotide sequence encoding said protein of interest under the control of a regulatory element, said regulatory element induced by the expression product of said isolated DNA..

57. The method of claim 55, wherein said protein of interest is a native protein.

58. The method of any one of claims 55 or 56, wherein said protein of interest is selected from the group consisting of a pharmaceutically active protein, antibody, industrial enzyme, protein supplement, nutraceutical, storage protein, an enzyme involved in oil biosynthesis, animal feed, and animal feed supplement.

59. The isolated DNA molecule of claim of any one of claims 4 to 7, wherein said isolated DNA molecule encodes a protein that is at least 70% homologous with the amino acid defined by SEQ ID NO:2.

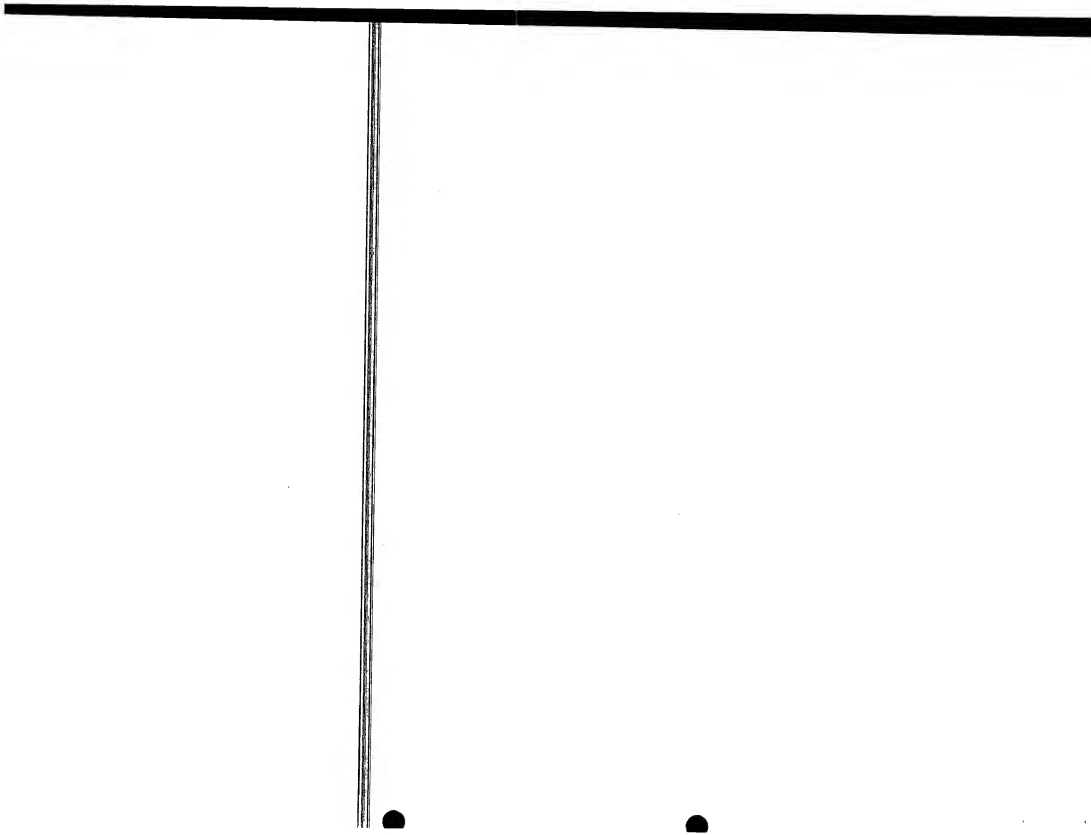
60. The isolated DNA molecule of claim of any one of claims 4 to 7, wherein said isolated DNA molecule encodes a protein that is at least 70% homologous with the amino acid defined by SEQ ID NO:4.

61 The isolated protein of claim 18, wherein said protein comprises from about 30 to about 541 amino acids of the sequence disclosed in SEQ ID NO:2

62. The isolated protein of claim 18, wherein said protein comprises from about from about 30 to about 561 amino acids of the sequence disclosed in SEQ ID NO: 4.

ABSTRACT OF THE DISCLOSURE

The present invention provides for a gene obtained during the induction of microspore embryogenesis. The protein encoded by this gene renders plant cells embryogenic, and increases the regenerative capacity of the plant cell. Also disclosed is the regulatory region of this gene and its use for directing the expression of a gene of interest within a suitable host cell.



In vivo development
In vitro development
embryogenic (32 °C)
non-embryogenic
(25 °C/32 °C)
gametophytic (< 25 °C)

Figure 1

Figure 2


```
BNM3A  GTTCATCTCTCTTCTTTAAGACCAAAACCTTTTTCTCCTCCTCTTCATGCATGAACCCTA   60
BNM3B  ------------------------------------------------------------

BNM3A  ACTAAGTTCTTCTTCTTTTACCTTTTACCAAGAACTCGTTAGATCACTCTCTGAACTCAA  120
BNM3B  ----------TTCTTCTTTTACCTTTTACCAAGAACTCGTTAGATCATTTTCTGAACTCGA   51
                 ************************************ * ********* *

BNM3A  TGAATAATAACTGGTTAGGCTTTTCTCTCTCTCCTTATGAACAAAATCACCATCGTAAGG  180

BNM3B  TGAATAATAACTGGTTAGGCTTTTCTCTCTCTCCTTATGAACAAAATCACCATCGTAAGG  111
       ************************************************************

BNM3A  ACGTCTACTCTTCCACCACCACAACCGTCGTAGATGTCGCCGGAGAGTACTGTTACGATC  240
BNM3B  ACGTCTGCTCTTCCACCACCACAACCGCCGTAGATGTCGCCGGAGAGTACTGTTACGATC  171
       ****** ********************* ************************************

BNM3A  CGACCGCTGCCTCCGATGAGTCTTCAGCCATCCAAACATCGTTTCCTTCTCCCTTTGGTG  300
BNM3B  CGACCGCTGCCTCCGATGAGTCTTCAGCCATCCAAACATCGTTTCCTTCTCCCTTTGGTG  231
       ************************************************************

BNM3A  TCGTCGTCGATGCTTTCACCAGAGACAACAATAGTCACTCCCGAGATTGGGACATCAATG  360
BNM3B  TCGTCCTCGATGCTTTCACCAGAGACAACAATAGTCACTCCCGAGATTGGGACATCAATG  291
       ***** ************************************************** *****

BNM3A  GTTGTGCATGCAATAACATCCACAACGATGAGCAAGATGGACCAAAGCTTGAGAATTTCC  420
BNM3B  GTAGTGCATGTAATAACATCCACAATGATGAGCAAGATGGACCAAAACTTGAGAATTTCC  351
       ** ******* ************** ********************* ************

BNM3A  TTGGCCGCACCACCACGATTTACAACACCAACGAAAACGTTGGAGATGGAAGTGGAAGTG  480
BNM3B  TTGGCCGCACCACCACGATTTACAACACCAACGAAAACGTTGGAGATATCGATGGAAGTG  411
       ************************************************  ********

BNM3A  GCTGTTATGGAGGAGGAGACGGTGGTGGTGGCTCACTAGGACTTTCGATGATAAAGACAT  540
BNM3B  GGTGTTATGGAGGAGGAGACGGTGGTGGTGGCTCACTAGGACTTTCGATGATAAAGACAT  471
       * ***********************************************************

BNM3A  GGCTGAGAAATCAACCCGTGGATAATGTTGATAATCAAGAAAATGGCAATGCTGCAAAAG  600
BNM3B  GGCTGAGAAATCAACCCGTGGATAATGTTGATAATCAAGAAAATGGCAATGGTGCAAAAG  531
       ************************************************** ********

BNM3A  GCCTGTCCCTCTCAATGAACTCATCTACTTCTTGTGATAACAACAACGACAGCAATAACA  660
BNM3B  GCCTGTCCCTCTCAATGAACTCATCTACTTCTTGTGATAACAACAACTACAGCAGTAACA  591
       ************************************************** ****** *****

BNM3A  ACGTTGTTGCCCAAGGGAAGACTATTGATGATAGCGTTGAAGCTACACCGAAGAAAACTA  720
BNM3B  ACCTTGTTGCCCAAGGGAAGACTATTGATGATAGCGTTGAAGCTACACCGAAGAAAACTA  651
       ** ***********************************************************

BNM3A  TTGAGAGTTTTGGACAGAGGACGTCTATATACCGCGGTGTTACAAGGCATCGGTGGACAG  780
BNM3B  TTGAGAGTTTTGGACAGAGGACGTCTATATACCGCGGTGTTACAAGGCATCGGTGGACAG  711
       ************************************************************

BNM3A  GAAGATATGAGGCACATTTATGGGATAATAGTTGTAAAAGAGAAGGCCAAACGCGCAAAG  840
BNM3B  GAAGATATGAGGCACATTTATGGGATAATAGTTGTAAACGAGAAGGCCAAACGCGCAAAG  771
       ************************************************ ************

BNM3A  GAAGACAAGTTTATTTGGGAGGTTATGACAAAGAAGAAAAAGCAGCTAGGGCTTATGATT  900
BNM3B  GAAGACAAGTTTATTTGGGAGGTTATGACAAAGAAGAAAAAGCAGCTAGGGCTTATGATT  831
       ************************************************************

BNM3A  TAGCCGCACTCAAGTATTGGGGAACCACCACTACTACTAACTTCCCCATGAGCGAATATG  960
BNM3B  TAGCCGCACTCAAGTATTGGGGAACCACCACTACTACTAACTTCCCCATGAGCGAATATG  891
       ************************************************************

BNM3A  AAAAAGAGGTAGAAGAGATGAAGCACATGACAAGGCAAGAGTATGTTGCCTCACTGCGCA 1020
BNM3B  AGAAAGAGATAGAAGAGATGAAGCACATGACAAGGCAAGAGTATGTTGCCTCACTTCGCA  951
       * ******* ************************************************ ****

BNM3A  GGAAAAGTAGTGGTTTCTCTCGTGGTGCATCGATTTATCGTGGAGTAACAAGACATCACC 1080
BNM3B  GGAAAAGTAGTGGTTTCTCTCGTGGTGCATCGATTTATCGTGGAGTAACAAGACATCACC 1011
       ************************************************************
```

```
BNM3A  AACATGGAAGATGGCAAGCTAGGATAGGAAGAGTCGCCGGTAACAAAGACCTCTACTTGG  1140
BNM3B  AACATGGAAGATGGCAAGCTAGGATAGGAAGAGTCGCCGGTAACAAAGACCTCTACTTGG  1071
       ************************************************************

BNM3A  GAACTTTTGGCACACAAGAAGAAGCTGCAGAGGCATACGACATTGCGGCCATCAAATTCA  1200
BNM3B  GAACTTTTGGCACACAAGAAGAAGCTGCAGAGGCATACGACATTGCGGCCATCAAATTCA  1131
       ************************************************************

BNM3A  GAGGATTAACCGCAGTGACTAACTTCGACATGAACAGATACAACGTTAAAGCAATCCTCG  1260
BNM3B  GAGGATTAACCGCAGTGACTAACTTCGACATGAACAGATACAACGTTAAAGCAATCCTCG  1191
       ************************************************************

BNM3A  AAAGCCCTAGTCTTCCTATTGGTAGCGCCGCAAAACGTCTCAAGGAGGCTAACCGTCCGG  1320
BNM3B  AAAGCCCTAGTCTTCCTATTGGTAGCGCCGCAAAACGTCTCAAGGAGGCTAACCGTCCGG  1251
       ************************************************************

BNM3A  TTCCAAGTATGATGATGATCAGTAATAACGTTTCAGAGAGTGAGAATAGTGCTAGCGGTT  1380
BNM3B  TTCCAAGTATGATGATGATCAGTAATAACGTTTCAGAGAGTGAGAATAATGCTAGCGGTT  1311
       ************************************************ **********

BNM3A  GGCAAAACGCTGCGGTTCAGCATCATCAGGGAGTAGATTTGAGCTTATTGCACCAACATC  1440
BNM3B  GGCAAAACGCTGCGGTTCAGCATCATCAGGGAGTAGATTTGAGCTTATTGCAGCAACATC  1371
       ************************************************* ********

BNM3A  AAGAGAGGTACAATGGTTATTATTACAATGGAGGAAACTTGTCTTCGGAGAGTGCTAGGG  1500
BNM3B  AAGAGAGGTACAATGGTTATTATTACAATGGAGGAAACTTGTCTTCGGAGAGTGCTAGGG  1431
       ************************************************************

BNM3A  CTTGTTTCAAACAAGAGGATGATCAACACCATTTCTTGAGCAACACGCAGAGCCTCATGA  1560
BNM3B  CTTGTTTCAAACAAGAGGATGATCAACACCATTTCTTGAGCAACACGCAGAGCCTCATGA  1491
       ************************************************************

BNM3A  CTAATATCGATCATCAAAGTTCTGTTTCGGATGATTCGGTTACTGTTTGTGGAAATGTTG  1620
BNM3B  CTAATATCGATCATCAAAGTTCTGTTTCAGATGATTCGGTTACTGTTTGTGGAAATGTTG  1551
       ************************** ************************************

BNM3A  TTGGTTATGGTGGTTATCAAGGATTTGCAGCCCCGGTTAACTGCGATGCCTACGCTGCTA  1680
BNM3B  TTGGTTATGGTGGTTATCAAGGATTTGCAGCCCCGGTTAACTGCGATGCCTACGCTGCTA  1611
       ************************************************************

BNM3A  GTGAGTTTGATTATAACCCAAGAAACCATTATTACTTTGCTCAGCAGCAGCAGACCCAGC  1740
BNM3B  GTGAGTTTGACTATAACGCAAGAAACCATTATTACTTTGCTCAGCAGCAGCAGACCCAGC  1671
       ********** ***************************************************

BNM3A  AGTCGCCAGGTGGAGATTTTCCCGCGGCAATGACGAATAATGTTGGCTCTAATATGTATT  1800
BNM3B  ATTCGCCAGGAGGAGATTTTCCCGCGGCAATGACGAATAATGTTGGCTCTAATATGTATT  1731
       * ******** ***************************************************

BNM3A  ACCATGGGGAAGGTGGTGGAGAAGTTGCTCCAACATTTACAGTTTGGAACGACAATTAGA  1860
BNM3B  ACCATGGGGAAGGTGGTGGAGAAGTTGCTCCAACATTTACAGTTTGGAACGACAATTAGA  1791
       ************************************************************

BNM3A  AAAAATAGTTAAAGATCTTTAGTTATATGCGTTGTTGTGTGCTGGTGAACAGTGTGATAC  1920
BNM3B  AATAATAGTTAAAGATCTTTAGTTATATGCGTTGTTGTGTGCTGGTGAACAGTTTGATAC  1851
       ** ************************************** ** ********* ******

BNM3A  TTTGATTATGTTTTTTTCTTTCTCTTTTTCTTTTTCCTTGGTTAATTTCTTAAGACTTATT  1980
BNM3B  TTTGATTATGTTTTTT--TTTCTCTTTTTCATTTTGTTGGTTAGTTTCTTAAGACTTATT  1909
       ***************  ************ **** ******* ****************

BNM3A  TTTAGTTTCCATTAGTTGGATAAATTTTCAGACT----------------------------  2014
BNM3B  TTTTGTTTCCATTAGTTGGATAAATTTTCGGACTTAAGGGTCACTTCTGTTCTGACTTCT  1969
       *** ***************************** ****

BNM3A  ------------------------
BNM3B  GTCTAATACAGAAAAGTTTTCAT                                        1992
```

Figure 3

4/9

BNM3A	MNNNWLGFSLSPYEQNHHRKDVYSSTTTTVVDVAGEYCYDPTAASDESSAIQTSFPSPFG	60
BNM3B	MNNNWLGFSLSPYEQNHHRKDVCSSTTTTAVDVAGEYCYDPTAASDESSAIQTSFPSPFG	
	********************** ****** ******************************	
BNM3A	VVVDAFTRDNNSHSRDWDINGCACNNIHNDEQDGPKLENFLGRTTTIYNTNENVGDGSGS	120
BNM3B	VVLDAFTRDNNSHSRDWDINGSACNNIHNDEQDGPKLENFLGRTTTIYNTNENVGDIDGS	
	** ****************** ********************************** **	
BNM3A	GCYGGGDGGGSLGLSMIKTWLRNQPVDNVDNQENGNAAKGLSLSMNSSTSCDNNNDSNN	180
BNM3B	GCYGGGDGGGSLGLSMIKTWLRNQPVDNVDNQENGNGAKGLSLSMNSSTSCDNNNYSSN	
	************************************* ****************** * *	
	repeat 1	
BNM3A	NVVAQGKTIDDSVEATPKKTIESFGQR**TSIYRGVTRHRWTGRYEAHLWDNSCKREGQTRK**	240
BNM3B	NLVAQGKTIDDSVEATPKKTIESFGQR**TSIYRGVTRHRWTGRYEAHLWDNSCKREGQTRK**	
	* **	
BNM3A	**GRQVYLGGYDKEEKAARAYDLAALKYWGTTTTTNFPMSEYEKEV**EEMKHMTRQEYVASLR	300
BNM3B	**GRQVYLGGYDKEEKAARAYDLAALKYWGTTTTTNFPMSEYEKEI**EEMKHMTRQEYVASLR	
	*** **************	
	repeat 2	
BNM3A	RKSSGFSRG**ASIYRGVTRHHQHGRWQARIGRVAGNKDLYLGTFGTQEEAAEAYDIAAIKF**	360
BNM3B	RKSSGFSRG**ASIYRGVTRHHQHGRWQARIGRVAGNKDLYLGTFGTQEEAAEAYDIAAIKF**	
	**	
BNM3A	**RGLTAVTNFDMNRYNVKA**ILESPSLPIGSAAKRLKEANRPVPSMMMISNNVSESENSASG	420
BNM3B	**RGLTAVTNFDMNRYNVKA**ILESPSLPIGSAAKRLKEANRPVPSMMMISNNVSESENNASG	
	** ***	
BNM3A	WQNAAVQHHQGVDLSLLHQHQERYNGYYYNGGNLSSESARACFKQEDDQHHFLSNTQSLM	480
BNM3B	WQNAAVQHHQGVDLSLLQQHQERYNGYYYNGGNLSSESARACFKQEDDQHHFLSNTQSLM	
	***************** **	
BNM3A	TNIDHQSSVSDDSVTVCGNVVGYGGYQGFAAPVNCDAYAASEFDYNARNHYYFAQQQQTQ	540
BNM3B	TNIDHQSSVSDDSVTVCGNVVGYGGYQGFAAPVNCDAYAASEFDYNARNHYYFAQQQQTQ	
	**	
BNM3A	QSPGGDFPAAMTNNVGSNMYYHGEGGGEVAPTFTVWNDN	579
BNM3B	HSPGGDFPAAMTNNVGSNMYYHGEGGGEVAPTFTVWNDN	
	**************************************	

Figure 4

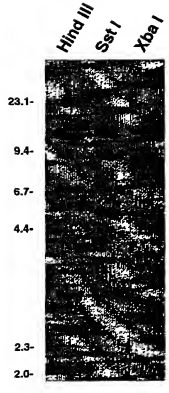


Figure 5

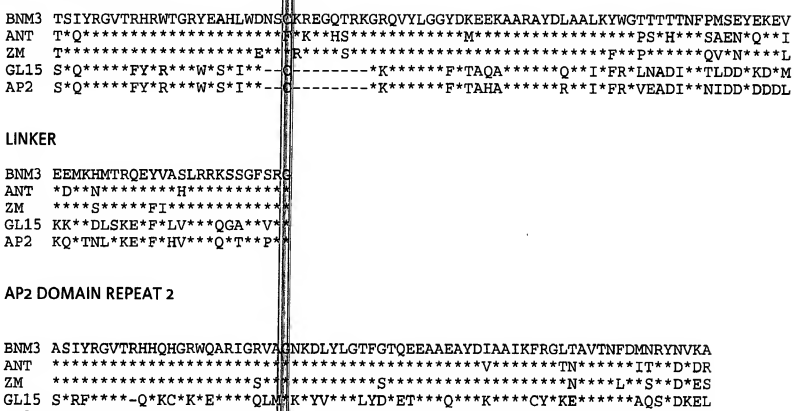


Figure 6

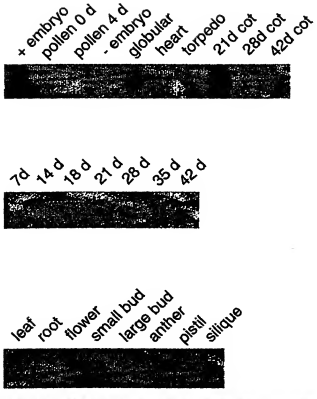
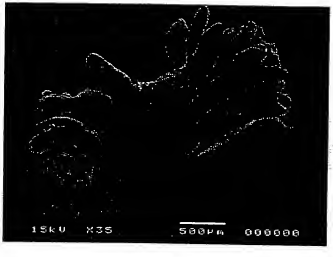

A
+ embryo
pollen 0 d
pollen 4 d
- embryo
globular
heart
torpedo
21d cot
28d cot
42d cot
B
7d
14 d
18 d
21 d
28 d
35 d
42 d
C
leaf
root
flower
small bud
large bud
anther
pistil
silique

Figure 7



e

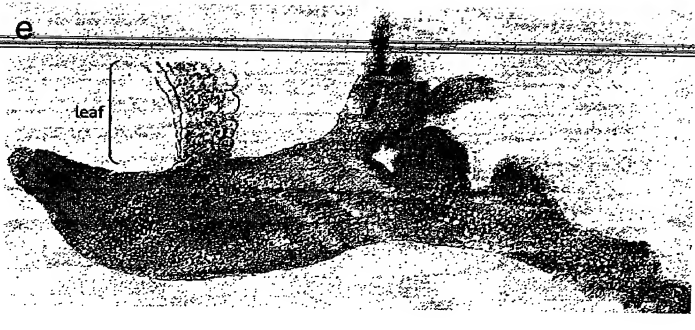


Figure 8

A
wild-type
transgenic
B
wild-type
transgenic
C
wild-type
transgenic
D
wild-type
transgenic